



IMPERIAL INSTITUTE
OF
AGRICULTURAL RESEARCH, PUSA.

ANNALS OF BOTANY

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AND OTHER BOTANISTS

NEW SERIES. VOLUME III

With twenty-four Plates and seven hundred and fifty-seven
Figures in the Text

OXFORD
AT THE CLARENDON PRESS

1939

OXFORD UNIVERSITY PRESS
AMEN HOUSE, E.C. 4
LONDON EDINBURGH GLASGOW NEW YORK
TORONTO MELBOURNE CAPE TOWN BOMBAY
CALCUTTA MADRAS
HUMPHREY MILFORD
PUBLISHER TO THE UNIVERSITY

PRINTED IN GREAT BRITAIN AT THE UNIVERSITY PRESS, OXFORD
BY JOHN JOHNSON, PRINTER TO THE UNIVERSITY

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Studies in Differentiation

VI. The Distribution of Calcium Malate and other Solutes in the Stems and Leaves of Succulent Compositae

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With fifteen Figures in the Text

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INTRODUCTION

THE work described here followed on that of Thoday and Evans (1932, 1933) on the distribution of calcium and phosphate and some other solutes in the tissues of *Kleinia articulata*. They found in the stem of this plant that soluble calcium is normally abundant, largely as malate, in the cell sap of the pith and inner cortex, the calciferous regions being sharply delimited from the non-calciferous regions; that soluble phosphates occur in close association with the vascular bundles; and that calcium and phosphate are strikingly complementary in their distribution. They also made a preliminary survey of the distribution of other important solutes.

¹ This paper embodies the substance of a thesis presented by the first-named author for the degree of M.Sc. in the University of Wales (Roberts, 1934).

The first object of the present work was an extension of this work on *Kleinia articulata*. The localization of inulin, the chief storage carbohydrate, was carefully investigated, and convenient methods for detecting both inulin and reducing sugar, from which presumably it must be formed, are described.

Whereas inulin is a storage product which can be easily mobilized, the significance of calcium malate is less obvious. Either it may act as a reserve material, or it may be merely a waste product, like the calcium oxalate crystals in the hypodermis. With the object of finding out whether it can be used as a food reserve, experiments were made on the sprouting and starving of cuttings.

A further examination of the distribution of solutes in *K. nerifolia* was then made. Finally a comparative study of stems and leaves of other succulent Compositae was undertaken.

A number of the plants were grown from cuttings supplied from Kew through the kindness of the Director. Thanks are also due to Miss A. J. Davey and Mr. N. Woodhead for their assistance in the supply of material.

METHODS

For the detection and localization of solutes the methods used were in the main those already detailed by Thoday and Evans (1932, 1933). Only supplementary notes need be added here.

The method employed by Patschovsky (1920), in connexion with the detection of oxalic acid in plant cells by aqueous FeSO_4 , for investigating the process of precipitation under the microscope, has been used to compare alcoholic with aqueous oxalic acid solutions as precipitants for calcium. To aqueous calcium nitrate in gelatine under a coverslip the reagent was run in and the progress of precipitation observed, the time taken for the precipitate to reach across the field of the microscope being noted. Precipitation always occurs in the weaker solution, whether in the gelatine or outside it, and with a stronger solution the progress of precipitation is more rapid. With alcoholic oxalic the progress was slower than with aqueous oxalic of equal strength. The undoubtedly advantage of alcoholic oxalic is therefore due to the expulsion of air and the rendering of the protoplasm permeable.

Experiments with calcium malate in the gelatine gave similar results. With 2 per cent. aqueous oxalic, precipitation began with the formation of small particles near the boundary. Later crystals, farther from the boundary, were larger; and of two forms in approximately equal numbers, probably trihydric (tetrahedral) and monohydric calcium oxalate respectively (compare Frey, 1929). In the succulent tissues under investigation the calcium oxalate precipitated by alcoholic oxalic appeared to consist of minute crystals of the monohydric form alone. The most convenient reagent was found to be 2 per cent. oxalic acid in 70 per cent. alcohol. Precipitation of calcium (in sections 0.3 or 0.5 mm. thick) is very rapid, no increase in density being detected after a minute; but sections are left several hours or overnight in the reagent to clear out the chlorophyll.

The precipitation of calcium malate in gelatine by alcohol (95 per cent. or absolute) produced milkiness but no distinguishable crystals.

Some observations suggest that a small part of the calcium malate present in sections may be precipitated as such by rapidly entering alcohol, and not as calcium oxalate, as in addition to the oxalate, which appears fawn-coloured by transmitted light, a darker, coarser precipitate is seen (especially in the rhizome of *K. articulata*) which disappears on washing with water. It is advisable therefore to follow the alcoholic oxalic by a brief immersion in aqueous oxalic before transferring to glycerine jelly.

Euparal has advantages as a mounting medium (Canada balsam renders the precipitate immediately invisible) as in a freshly prepared slide the precipitate is clearly visible and the cell walls less prominent than in glycerine jelly; but after some weeks the precipitate vanishes. It can be revealed again by transferring through euparal essence to absolute alcohol.

Inulin. Inulin has been shown to exist in more than one form. Tanret (1893), from the Jerusalem Artichoke, separated inulin, pseudo-inulin, and inulenin. Thaysen, Bakes, and Green (1929) isolated from autumn tubers of Jerusalem Artichoke true inulin, which was insoluble in 55 per cent. alcohol, and pseudo-inulin, which was insoluble in 85 per cent. alcohol, but dissolved in the lower strengths. As the inulin found in the Kleinias examined was precipitated by 55 per cent. alcohol it must consist chiefly, at any rate, of true inulin.

In absolute alcohol precipitation appears to be complete within a few minutes. The lower strengths of alcohol precipitate inulin more slowly, but even 10 per cent. caused precipitation after some hours, except when the amount present was small. In all strengths, boiling greatly accelerated the process.

The appearance of inulin in the cells is different when it is precipitated by different strengths of alcohol. Alcohols lower than about 70 per cent. give large, round or oval sphaerocrystals (usually 3–6 in each cell). Higher strengths (80–90 per cent.) give smaller globules, more numerous in each cell. Absolute alcohol and 95 per cent. give granular masses (presumably composed of very minute sphaerocrystals aggregated together). If a section is boiled in absolute alcohol (to remove air) after the large sphaerocrystals have been produced by a lower strength of alcohol, the crystals remain large and distinct.

Taking all these factors into consideration, the best way of showing inulin is to leave sections overnight in 70 per cent. alcohol, as the sphaerocrystals thus produced are characteristic and unmistakable. If, however, quicker results are needed, the sections may be placed in cold or boiling absolute alcohol and examined at once. The precipitated inulin can be stained if desired by leaving overnight in alcoholic light green.

Euparal is an excellent mounting medium for inulin preparations; glycerine jelly can also be used.

When calcium malate is present in the cells, the brownish precipitate

formed by alcohol may interfere with the examination of inulin. The precipitate of calcium malate may be removed by washing the section (after it has been in alcohol) in water. The malate disappears, and the inulin partially dissolves, but remains in the cells, and may be precipitated again by replacing in alcohol.

Inulin may be precipitated by the reagent for calcium (alcoholic oxalic acid) and it is sometimes convenient to have calcium and inulin shown on the same section. But if left too long in acid, the inulin is hydrolysed. (This fact is utilized in preparing a calcium preparation free from inulin.)

Phosphate. Preparations made by Macallum's ammonium molybdate and phenyl hydrazine method are best mounted in Canada balsam. In euparal after a few weeks the blackened phosphate granules fade to yellow, the colour they had before treatment with phenyl hydrazine. If such sections are transferred to alcohol and again treated with phenyl hydrazine they once more turn black. This suggests that the blackening is a superficial effect and the granules are merely coated with the black lower oxide of molybdenum.

Reducing sugar. Thoday and Evans (1933) were unable to detect reducing sugar in *Kleinia articulata* microchemically by the osazone method. It has, however, been found possible to demonstrate its presence and localize it by means of Fehling's solution. Two methods have been employed:

(i) The section is dropped into boiling Fehling's solution. After about half a minute it is transferred to water in a watch glass. The macerating influence of the reagent makes the section difficult to handle without disintegration. This can, however, be counteracted by treatment with lime water for ten minutes (to allow the re-formation of calcium pectate in the middle lamellae), after which the section can easily be handled, dehydrated, and mounted in Canada balsam.

(ii) The section is placed on one end of a slide, water blotted off and a couple of drops of Fehling's solution added, and the slide gently heated over a flame till the reagent begins to boil. The section is then washed in water and either examined in water or glycerine or dehydrated and mounted in balsam. In most cases sections treated in this way were quite easy to handle.

Especially for permanent preparations in Canada balsam, it was found advantageous to counterstain the sections as the unstained walls become in time very indistinct. Light green (in alcohol or clove oil) gave the best results. The precipitate, light red or orange as seen with the naked eye by reflected light, appears under the microscope as dark red copper particles more or less crowded together in the cells.

In order to test whether the location of the copper precipitate gave a true picture of the distribution of sugar in the tissues, or whether it diffuses during treatment with the reagent, longitudinal sections were cut. One of these was treated as described, another was cut longitudinally into strips and each strip tested separately. There was no appreciable difference in the distribution indicated.

With sections of *K. neriifolia* and *K. articulata* stems, after boiling in Fehling's solution, a whitish line was observed following the wavy outline of the bundles outside the bundle zone and also in the outer pith inside the bundle zone. A similar line was also present round each leaf-trace bundle in the cortex. Tests showed that it was also formed in caustic soda or potash alone. As it appeared invariably to occur at the boundary between tissue containing soluble calcium salts and tissue containing soluble phosphates, it was thought probable that calcium phosphate, insoluble in alkaline solution, had been precipitated at the boundary. Acetic acid dissolved it; but application of the molybdate test did not reveal any concentration of phosphate there. The nature of this precipitate therefore remains to be discovered.

Another fact which revealed itself when sections were boiled in soda and potash (10 per cent. aqueous) was that in soda there was considerable shrinkage of the tissues, sometimes by as much as 70 per cent. of the original dimensions, while in potash there was far less shrinkage or none at all. This contrast was still observed when solutions of equivalent concentrations were used. Separate treatment of pith and outer tissues from longitudinal sections showed that the contraction occurred in the pith but not appreciably in the cortex.

A typical experiment with *K. neriifolia* pith gave the following results (measurements of tracings with micro-projection apparatus):

(a)	initial dimensions 10·5 × 21 cm. = 220·5 sq. cm.
	after boiling in 10 per cent. NaOH	. 5·5 × 12·5 cm. = 68·7 sq. cm.
(b)	initial dimensions 11·5 × 23 cm. = 264·5 sq. cm.
	after boiling in 14 per cent. KOH	. 10·0 × 22 cm. = 220 sq. cm.

Longitudinal sections of the pith of *Juncus* sp., Elder, and Jerusalem Artichoke all contracted considerably in 10 per cent. NaOH, more strongly after boiling, whereas in 14 per cent. KOH they showed little or no change, so that this phenomenon is not peculiar to the succulents though the shrinkage is greater in them. It might be attributed to the removal of pectic constituents from the cell walls; but it is not clear why in this case the effects of potash and soda should contrast so strongly.

Chloride. The localization of chloride by silver nitrate is not satisfactory in the presence of malates, although silver chloride rapidly turns purple in the light and silver malate changes more slowly to a brownish colour. The indications given by this method differed from the results described by Thoday and Evans (1933), who relied mainly on analysis of zones separated with a scalpel, of necessity only roughly. Attempts were therefore made to obtain information concerning the distribution of chlorides by other means, depending on removal of malate along with other organic substances by careful incineration. This was first tried with a transverse section on a slide, with indifferent success.

Fairly thick longitudinal sections were next tried. They were separated into three zones: cortex, bundle zone, and pith. Equal weights of these zones were taken, placed in three crucibles, and heated strongly until reduced to a

white ash. The ash from each zone was dissolved in one or two c.c. of nitric acid, and placed in a watch glass. Equal volumes of silver nitrate were added to each, and the amount of chloride present was judged by the extent of milkiness formed. This method only allowed of a rough comparison, and required an inconveniently large amount of material.

TABLE I

Tissue.	Fresh weight in gm.	c.c. of AgNO_3 used.	Gm. chlorine in 100 gm. fresh wt. (average for each tissue).
Pith	0.276	1.2	
	0.27	0.9	
	0.28	0.95	0.113
	0.27	0.7	
	0.32	0.92	
Cortex	0.325	0.55	
	0.36	0.55	
	0.27	0.35	0.048
	0.34	0.4	
	0.3	0.4	
Bundle zone	0.23	0.25	
	0.255	0.25	
	0.33	0.30	0.038

Incineration on a platinum wire allowed the use of much smaller quantities of material. Longitudinal sections were taken (about 1.5 cm. long and 0.5-1.0 mm. thick) and cut into strips (about 2 mm. wide). Each strip in turn was attached to a platinum wire and carefully and slowly incinerated by passing it through a micro-flame. Care was taken not to burn the strips too quickly as chloride might volatilize. The white ash was dissolved in a drop of nitric acid in a watch glass and a drop of silver nitrate added. This method was found to be quite quick, and useful for rough comparative purposes. Attempts to refine it by collecting the precipitate as a column in a capillary tube were unsuccessful.

Micro-volumetric analysis was then tried. For this rather larger quantities of material were necessary. Several fairly thick longitudinal sections were cut, and divided into separate zones as carefully as possible. Enough strips of one tissue to make up about 0.2 or 0.3 gm. were weighed, incinerated, and dissolved in about 2 c.c. of nitric acid in a small glass tube. Chalk was added to neutralize the acid, two drops of 7 per cent. potassium chromate were added as indicator, and the solution titrated with N/100 silver nitrate. The data given in Table I, which were obtained with a branch of *K. nerifolia*, show a reasonable consistency for each tissue and definite differences between the tissues.

Adding excess of AgNO_3 and titrating the excess against standard KCNS was also tried, but this method involved too much loss by filtration.

Extraction by absolute alcohol instead of incineration (calcium malate being insoluble in alcohol) gave values about double those in Table I. This

probably means that other salts of malic acid are extracted by the alcohol, or that calcium malate itself is slightly soluble. Alternatively, some loss of chloride may occur by volatilization during incineration, but it is highly improbable that if this were so to an appreciable extent the results would be as consistent. Both methods placed the tissues in the same proportional relation as regards chloride content.

RESULTS

Kleinia articulata Haw.

Calcium. In the outer pith and inner cortex of the stem the calcium exists chiefly as malate, precipitated by alcohol. In the central pith the proportion of calcium nitrate, precipitated only by oxalic acid, is higher. Total calcium does not vary greatly from the tip to the base of a mature joint, but the tip has appreciably less calcium malate than the base.

In the constricted part of a stem formed by a check to growth the bundle zone was much wider than elsewhere, and the cortex narrower. Calcium was present only in the pith and was more abundant towards the centre.

At the extreme base of a branch just above the constricted articulation there was no calcium in the pith and only a little in the cortex. Calcium was present in the pith but not the cortex of the main stem at the junction, and just below the junction there was a group of cells in the outer pith containing clusters of calcium oxalate crystals.

In *oetiolated shoots* the amount of calcium found was very small, and it occurred only in the pith cells.

In the *rhizome* (Fig. 1) calcium is found throughout the pith, to within one or two rows of the bundle zone, largely as malate. The central pith cells sometimes, but not always, have less calcium than the peripheral cells. Occasionally calcium is detected in the cortex outside the wide inulin zone (see below).

Phosphate. While always present in the bundle zone and round leaf-trace bundles and resin ducts in the cortex, phosphate frequently extends from the bundle zone to the outer pith, and is sometimes found in the outer cortex. When very abundant (as in plants given extra phosphate) it occurs throughout the cortex and outer pith. There is usually less phosphate in young stems; occasionally none is precipitated.

In etiolated stems phosphate is very scarce.

Where a normal branching stem was examined no phosphate was found at the point of junction of stem and branch.

In the rhizome there is less phosphate than in the stem; it encroaches on the pith but is rarely found in the cortex.

Inulin. When present in *aerial stems* inulin may occur in few, or be abundant in all the parenchymatous cells round and between vascular bundles, but is never found in the cortex or pith. Cuttings which when planted contained very little inulin had in four months produced roots and young shoots, and

stored inulin in abundance in the bundle zone and also in the wound phellogen (Woodhead, 1934).

The rhizome stores large quantities of inulin (Fig. 1); it is located chiefly in the bundle zone but usually spreads also to the outer pith and a great part of the cortex, of which sometimes only the two or three outermost layers of cells do not show it.

Rhizomes vary considerably in diameter from one part to another. In the narrow parts only a little inulin was found, confined to the bundle zone; in the thickest parts it was abundant, from cortex to outer pith. This corresponds fairly closely with Holm's observations (1931) on the localization of inulin in the roots of Compositae and the correlation between diameter and inulin storage.

Reducing sugar. Great variation has been noticed in *Kleinia articulata* stems with regard to their reducing sugar content.

Young, leafy stems usually gave an abundant precipitate, located invariably in the pith. Longitudinal sections through a whole young joint showed most towards the middle, and less at the base and apex. Some young stems, however, showed no trace of sugar. Old stems very often had no sugar; but sometimes it was present in the cells of the inner cortex and bundle zone, but *not* in the pith. In these cases it was usually found on one side of the stem only, and there was some indication that this was more frequently the shaded side, but no reliable conclusion can be drawn on this point.

When extracted juice of old stems was tested with Fehling's solution no sugar was detected, even when the juice was obtained from stems which gave a positive result microchemically. Sugar could easily be detected in juice extracted from young stems.

In the peduncle of a flowering stem, reducing sugar was found in abundance in the pith. In this case sugar was also present in most of the main stem, but at the part where stem merged into peduncle none was found.

Potassium. Potassium is present in all aerial stem tissues; there is usually less in the bundle zone and outer pith than in the central pith and cortex, and there is more in young stems than in old. In the rhizome none was detected.

Chlorides. Both microchemical observations, so far as they went, and qualitative tests with separated tissues indicated that the pith contained most chloride, and the bundle zone least. Using the quantitative microtitration method this was confirmed. The following data were obtained by the incineration of separated parts of sections:

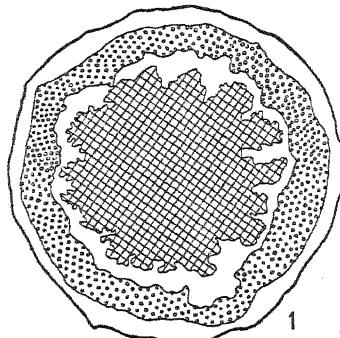


FIG. 1. *Kleinia articulata*: rhizome. Transverse section showing distribution of calcium (cross hatched) in the pith and inulin (rings) in a broad zone including the bundles.

Tissue.	Weight in gm.	Amount of N/100 in 100 gm. fresh AgNO ₃ used.	Gm. of chlorine wt.
Cortex . .	0·13	0·35 c.c.)	0·088
	0·13	0·30 c.c.)	
Bundle zone . .	0·13	0·18 c.c.)	0·040
	0·13	0·11 c.c.)	
Outer pith . .	0·13	0·37 c.c.)	0·092
	0·13	0·32 c.c.)	
Inner pith . .	0·13	0·52 c.c.)	0·140

These results were obtained in April 1932. In March 1933 some further determinations were made, after extracting the tissues with alcohol or with hydrogen peroxide, as incineration involved some possibility of loss. The results given are for different samples of tissues of the same stems by the two methods of extraction.

Tissue.	Gm. Cl. in 100 gm. fresh weight. Hydrogen peroxide extraction.	Alcohol extraction.
Cortex . .	0·134	0·14
Bundle zone . .	0·22	0·17
Pith . .	0·24	0·20

The agreement between the two columns is not satisfactory, but like the previous results they indicate a higher chloride content in the pith. When these data are taken along with those of Thoday and Evans, some variation in the distribution of chlorides with age and season appears probable.

Nitrates. All tests for nitrates confirmed Thoday and Evans's statement that the pith was rich in nitrates, and the other tissues had progressively less, up to the hypodermal layers, which gave only a very slight reaction.

Oxidases. When guaiacum tincture was applied to a section of the stem a blue colour was first seen round the bundles. After addition of hydrogen peroxide the whole of the bundle zone became blue, indicating the presence of indirect oxidases, but no reaction was observed in the pith or cortex.

Experiments on mobilization of reserves in cuttings.

Sprouting of cuttings in the light. If calcium malate functions as a food reserve, its utilization might be expected during the sprouting of lateral branches and production of roots from cuttings. To see whether any transfer from the parent stem could be detected by the microchemical methods already employed, mature leafless joints of *K. articulata*, selected for uniform appearance, morphology, and past history, were cut into pieces about 4·5 cm. long. Sections cut from the two ends of each piece (except in the case of apical pieces) were examined for calcium, calcium malate, inulin, phosphate, potassium, and reducing sugar, and the preparations kept for reference. At intervals of a few weeks sample cuttings were taken for examination.

The changes that occur at cut surfaces have already been described by Woodhead (1934). Beyond these changes the results were negative.

Sprouting cuttings showed roughly the same amount of malate as was originally found, and there was no evidence of any decrease to indicate a transference of calcium malate to roots or lateral shoots.

Among the incidental observations the following points may be mentioned:

The phosphate content, initially normal, diminished considerably during rooting and sprouting in the bulk of the cuttings. (In many cuttings there was an accumulation of phosphate in the cells of the wound periderm (cf. Woodhouse 1934).)

No change was observed in the amount or distribution of potassium. It was noticed that potassium was more abundant when phosphates were scarce, and vice versa. Few of the cuttings showed any reducing sugar at the start, but it appeared later in the cortex near the base. Sugar was abundant in the pith of nearly all the young lateral branches (cf. p. 8).

Most of the cuttings showed little or no inulin at first and what little was present, in the cells between the bundles, soon disappeared. It was first seen again after five months, present in abundance at the base of a cutting, in the bundle zone and in the periderm, less towards the apex. After that it was found similarly distributed in all the cuttings, whether they bore leafy sprouts or not. This was in April, and may have been a seasonal effect.

Cuttings in the dark. Root-formation was almost entirely inhibited in darkness. So long as the tissues remained healthy the distribution of calcium and of calcium malate showed little if any change. After about fifty days at room temperature (17° C.), sometimes sooner in individual cuttings, injection of the air spaces with sap became evident. In injected parts alcohol precipitated no calcium malate. A cutting, which showed injection for about 2 cm. from one end, while the other end appeared firm, was found on sectioning to have the outer pith injected throughout, but the injected zone narrowed towards the externally normal end. In this case the cutting had initially been rich in calcium and calcium malate; now, after fifty days, no malate precipitated in the fully injected end, and the amount had diminished in the rest of it.

Starvation at 30° C. In order to shorten the period of starvation, cuttings were exposed in the dark to a temperature of 30° C. in an incubator. Half of these had the cut ends dipped in paraffin wax to prevent escape of juices; these remained healthy, but showed no wound reaction. On the seventh day one cutting showed injection. By the twelfth day six others showed injection and three still looked healthy. As before, no malate was precipitated by alcohol in injected parts. The expressed juice, moreover, had a high pH — 8.0–8.5 as compared with 5.6–6.2 in uninjected parts. Phosphate was found throughout the injected pith, and the amount appeared to be greater than that initially present. What inulin and sugar had been present had disappeared.

Injection began earlier at that end of a cutting containing more calcium, and, in the pith, in the outer zone of highest calcium malate concentration.

In connexion with the observations on starvation the respiration was

followed by means of a compensated manometric apparatus for measuring the volume of oxygen absorbed, similar to that described by Thoday (1932). At 30° C. the rate of oxygen intake first fell, reached a minimum about the fifth day, from which it rose after the eighth day. This rise in oxygen intake was found to be associated with, and was used as an index of, the beginning of injection.

Conclusions. These experiments have not yielded any evidence of the utilization of calcium malate as a food reserve in the production of adventitious roots or the sprouting of axillary buds, nor any clear indication of malate being drawn upon, even during starvation, until the cells approach the stage of exhaustion when the protoplasm fails to retain the sap-solutes.

Results obtained by microchemical tests are, however, only rough guides to quantities and concentrations, and the indications given by them must be followed up by quantitative investigations. This applies not only to the negative conclusions regarding the calcium malate but also to the apparent increase of phosphate when the starved tissues become injected.

If calcium malate is not translocated to growing roots and sprouts, the disappearance of it from the neighbourhood of a wounded surface should perhaps be regarded as a clearance in preparation for periderm formation rather than its utilization; or as a consequence of the changed conditions which accompany a return to meristematic activity.

Kleinia nerifolia Haw.

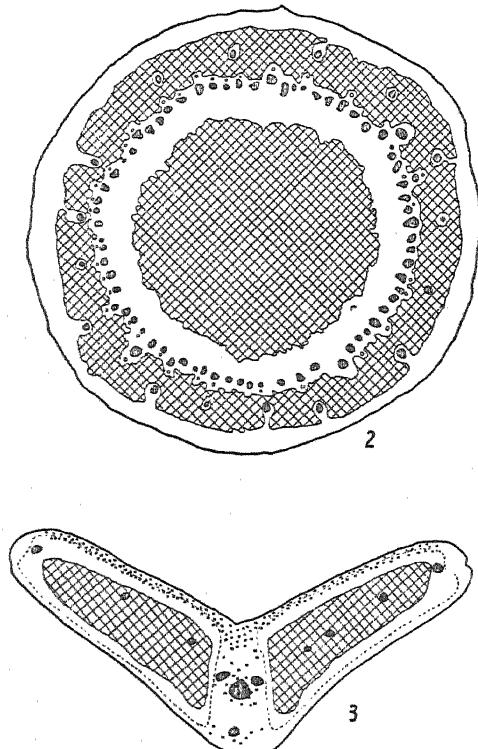
The plant used was about a metre high, the stem 3 cm. in diameter in the oldest part, the youngest joints 1 cm. The latter bore numerous linear sessile leaves, 5–10 cm. long, in a close spiral. The stem is of much firmer consistency than that of *K. articulata*; in transverse section (Fig. 2) the bundles are more numerous, the pith cells are smaller and more closely packed. Two rings of small resin ducts are seen, one just outside the bundles, the other in the outermost cortex. Chloroplasts are confined to the outer cortex, and are not numerous.

Fig. 2 represents a transverse section of a young leafy joint about 1 cm. in diameter and shows the distribution of calcium, which, as in *K. articulata*, is present in the cell sap in the pith and in the inner cortex. In the pith, however, outside the calcium zone and sharply delimited from it, is a zone about ten cells wide in which no calcium is precipitated by the reagent. This zone was not distinguished in the earlier account (Thoday and Evans, 1932). In each cell of this zone there is a small aggregate of calcium oxalate crystals (separate from the nucleus), already present before treatment. The cortical calcium zone, too, is wide, and continuous, except for the passage of leaf-trace bundles through it. The outer cortex, of six or seven layers of cells, the bundle zone, and a patch of cells round each leaf-trace bundle and resin canal, show no calcium.

The distribution of calcium malate, as precipitated by alcohol, appeared

identical in the cortex with that of calcium; but in the pith it was rather scanty. Thus while the bulk of the calcium in the cortex is probably in the form of malate, in the pith it is only partly malate and partly some other salt, probably nitrate, at this stage.

In an older joint (third from the apex) the outer zone of the pith was only



Figs. 2 and 3. Fig. 2. *Kleinia nerifolia*: stem. Transverse section showing distribution of calcium (cross hatched). Bundles are shaded; small rings are resin ducts. Fig. 3. *K. nerifolia*: leaf. Transverse section showing distribution of calcium (cross hatched) and phosphate (dotted). Bundles shaded; the broken lines indicate the outer boundaries of the chlorenchyma.

four or five cells wide and not all of them contained calcium oxalate crystals. Whether this means encroachment of calcium on cells not originally storing it, or merely attenuation of the calcium oxalate zone due to increase in diameter of the stem, is not clear. A larger proportion of sap-calcium in the pith was in the form of malate, which was now abundant in the outer part of the calcium-storing region. The calcium malate in the cortex showed no sign of any diminution (contrast *K. articulata*, see Thoday and Evans, 1932, pp. 789–90).

Phosphates occupy a wider zone in the stem of *K. nerifolia* than in that of *K. articulata*, corresponding to the calcium-free zone, from outside the resin

ducts of the bundle zone, through the bundle zone itself into the outer zone of the pith (see Thoday and Evans, 1932, p. 793, Fig. 2). It also occurs round each leaf-trace bundle. Phosphates and calcium appear therefore not to overlap at all.

Potassium was distributed irregularly in inner cortex, bundle zone, and pith of the young stem, but the older stems showed none. Nitrate, as in *K. articulata*, was at a maximum in the pith, from which it diminished in concentration towards the outermost layers where there was very little or none.

The distribution of chlorides was very similar to that in *K. articulata*. The titration methods gave, per 100 gm. fresh weight: after incineration, pith 0.113 gm., bundle zone 0.038 gm., cortex 0.048 gm.; by alcoholic extraction, in another part of the same stem, pith 0.21 gm., bundle zone 0.066 gm., cortex 0.101 gm.

Young stems always showed abundant reducing sugar in the pith, its distribution corresponding roughly with that of calcium. The zone of copper precipitate stopped within a few cells of the bundle zone, and none was detected outside it, in bundle zone or cortex. Older stems showed even more reducing sugar. So abundant was it that when sections were heated with Fehling's solution on a slide, some escaped from the cells and precipitated copper outside the section. The precipitate was densest in the centre of the pith, but extended right through the pith, in some cases into the bundle zone and inner cortex. None was found in the outer cortex. The abundance and persistence of reducing sugar in the pith of *K. nerifolia* contrasts markedly with its temporary presence in that of *K. articulata*.

No inulin was found in the young leafy joints. In the older joints it was constantly abundant in the bundle zone, the outer limit of its occurrence lying between the bundles and their resin canals, its inner limit in the extreme periphery of the pith.

Determination of the pH of expressed sap by the B.D.H. Capillitor gave results practically the same as for *K. articulata*. A young joint gave for pith 5.7, cortex 4.4; an older joint 5.8 and 4.6, i.e. slightly less acid, but the differences are doubtfully significant.

Direct oxidases were confined to the bundle zone, but indirect oxidases were generally distributed.

The leaf of *K. nerifolia* is hardly to be described as succulent, though it is thick and rather fleshy. Except for a band of colourless tissue along the midrib and narrow bands along the margins the bulk of the tissue is chlorenchyma. The distribution of calcium and phosphate is nevertheless of interest (Fig. 3). They do not overlap. Calcium is distributed in moderate amount through the green mesophyll, but not to its limits. Phosphate is scanty, but occurs in some of the colourless cells of the midrib and extends to a thin layer of cells along the upper surface of the leaf. No precipitate of calcium malate was obtained with alcohol, nor was any inulin detected.

Other Succulent Compositae

Some obvious lacunae in the following observations were due to the restricted amount of material available at the time. It is hardly necessary to say also that negative results are not to be stressed unduly. More extended investigation of individual species would doubtless reveal considerable variation in detail.

Kleinia repens Haw.

The plant selected had an erect stem, about 10 cm. high and 0.5 cm. in diameter, bearing spirally arranged leaves at intervals of less than 1 cm. The mature leaves were about 5 cm. in length, thick and succulent, and had a wide, shallow groove on the upper surface. Both leaves and stems were of a distinctly bluish colour, due to anthocyanin in the hypodermis. Lateral branches arose in the axils of some of the upper leaves.

Leaf. In the leaf there was a large central water-tissue of big colourless cells with thin walls. The surrounding green tissue was relatively narrow, and was broken up into bands by colourless parenchyma around the principal bundles, with their associated resin ducts (Fig. 8, p. 19).

The amount of calcium present differed greatly in different leaves, and in different parts of the same leaf. Some leaves showed no calcium, but in most of those examined calcium was precipitated in some of the water-storage cells (chiefly the outer ones), though never in every cell. In most sections about 50 per cent. or fewer of the water-storage cells contained calcium precipitate. In one section only three or four cells in a group had calcium. When sections through different parts of the leaf and longitudinal sections through the whole leaf were examined, it was noticed that the calcium content diminished towards the apex.

The leaves differed very much as to phosphate content, some having a large quantity, others very little or none. The phosphate occurred in the water-tissue only, and was much more abundant in the apical half of the leaf, where the calcium content was less.

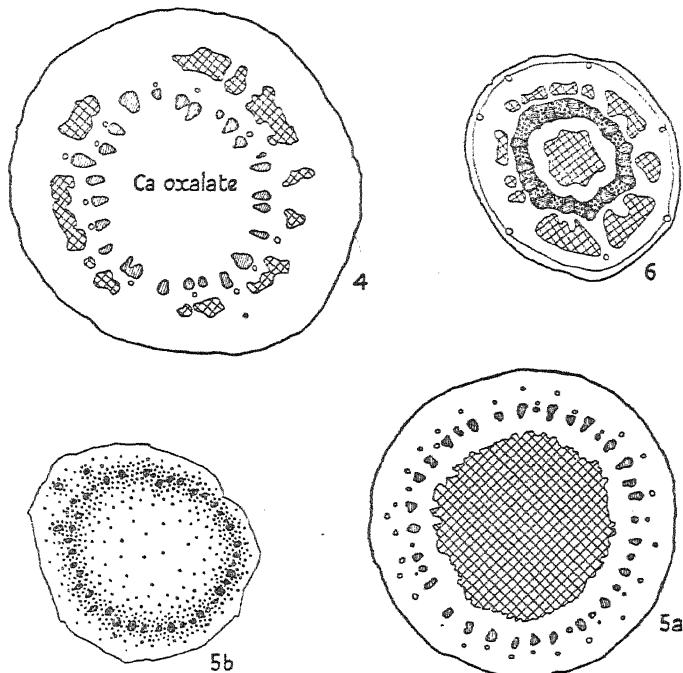
The tests for potassium, inulin, and oxalic acid gave negative results.

Stem. All the pith cells of the stem contained clusters of calcium oxalate crystals similar to those in the outer pith of *K. nerifolia*. Soluble calcium was confined to groups of cells of irregular size and shape in the inner cortex. (Fig. 4). The precipitate with alcohol was less than that with oxalic acid, indicating that not all the calcium was present as calcium malate.

The reaction for nitrate was given at once in the outer tissues, not at all in the pith. Different stems varied with regard to their phosphate content. In one case only a few particles were detected, in the bundle zone. In other stems phosphate was abundant in the bundle zone and round leaf-trace bundles, and a moderate amount was also present in all the pith cells (less at the centre). The results for potassium, inulin, and sugar were negative.

Kleinia aizoides D.C.

This species was somewhat similar in habit to *K. repens*, but the stem in some plants grew much longer, and when supported attained a height of more than a foot. The stem was delicate, 0·5 cm. in diameter, smooth, and pale



Figs. 4–6. Transverse sections of stems. Sap-calcium cross hatched; bundles shaded; rings outside bundle zone are resin ducts. Fig. 4. *K. repens*: calcium in inner cortex; pith cells contain calcium oxalate. Fig. 5. *K. aizoides*: a, calcium in pith only; b, smaller stem showing phosphate (dotted). Fig. 6. *K. radicans*: calcium in pith and inner cortex; inulin (rings) in bundle zone.

green in colour, and bore leaves spirally at intervals of about 2 cm. The leaves were 5–7 cm. long, narrowly cylindrical, and tapered to a fine point at the apex. Lateral branches arose in the axils of some of the leaves.

Leaf (Fig. 9). The section of the leaf was oval. A large water-tissue was surrounded by a narrow band of chlorenchyma, in this species continuous. The vascular bundles were on or near the inner border of the green tissue. Opposite each bundle, in the outer chlorenchyma, was a small duct with a ring of regular, colourless cells round it.

Calcium was abundant in the water-tissue, but in most sections there was a central patch devoid of it. Also the cells immediately inside the ring of bundles had no calcium. It was entirely absent from the chlorenchyma.

Phosphate had an irregular and scanty distribution. In some sections it was

found round the bundles, and in others a little was present just inside the bundle zone. In leaves examined several months later (Feb. 1933) no phosphate was found.

An irregular ring of cells just inside the green tissue gave precipitate when tested for oxalic acid. Potassium was present in moderate amounts in the centre of the leaf, and decreased towards the chlorenchyma, where none was precipitated.

Stem. When cut the stem exudes a thick white milky juice. The pith is large, the cortex narrow, with chlorophyll concentrated in the outer half and resin ducts in the inner outside most of the bundles.

Calcium (Fig. 5a) was abundant in the cells of the pith, except in the peripheral layers: the boundary was sharp, and no calcium was precipitated outside it. All the cells had about the same amount of precipitate (unlike *K. articulata*, where there is usually more calcium at the edge than at the centre). Calcium malate was rather less in amount than total calcium, especially at the centre, where it was scarce. The calcium at the centre of the pith was therefore chiefly calcium nitrate. Nitrate was abundant everywhere.

The amount of phosphate present varied in different specimens. In one case it was abundant in the bundle zone, inner cortex, and outer pith, and there was also a little throughout the remainder of the pith (Fig. 5b). In a thicker stem there was much less phosphate and it was located only in the bundle zone and outer pith. The tests for potassium, inulin, and sugar gave negative results.

Kleinia radicans Haw.

This plant has a slender prostrate stem, pinned to the ground at intervals by adventitious roots. The leaves were 2–3 cm. long, with a shallow groove on the upper side, making them heart-shaped in section. They were arranged spirally at intervals of 2 or 3 cm. on the stem, but turned vertically by curvature of the narrowed base. The apex of the stem, with the two or three youngest leaves, curved upwards.

Leaf. The leaf was characterized by an extensive water-tissue of very large cells, and a narrow band of chlorenchyma, which was not, however, limited sharply from the water-tissue. The cell size increased and the density of chloroplasts diminished from the outside layer of chlorenchyma to well within the bundles; only a relatively small zone in the centre was quite colourless, but it extended at the groove to the surface. In the extreme outer cortex were resin ducts, and the cells round these, together with all the cells of the epidermis and collenchymatous hypodermis, were colourless.

Calcium was found only in the water-tissue. This zone was rich in calcium, and was sharply defined, its boundary following the inner margin of the bundle zone (Fig. 10). Oxalic acid was found in a band of cells between the vascular bundles and the calciferous zone. Phosphate and potassium were not found.

Stem. The stem differed from most of those examined in that it had a

relatively small pith and wide cortex. The ring of bundles was rather irregular and the individual bundles large. There were a few resin ducts in the outer cortex. The epidermis and two or three rows of regular cells constituting the hypodermis were colourless, and chlorophyll was chiefly present in irregular patches of cells in the outer cortex (Fig. 6).

There was a well-defined calcium zone in the pith, leaving the peripheral pith cells free; and several irregular patches of cells in the inner cortex also contained calcium.

The stem was rich in inulin, which was stored in the bundle zone. The sphaerocrystals showed very clearly in an oxalic-acid-in-alcohol preparation (Fig. 6). Phosphate was abundant in all tissues. It was most abundant in the bundle zone, and least in the outer cortex. The phenomenon of phosphate particles outlining the cell walls was particularly well shown in this stem.

Othonna carnosa Less.

These plants were very similar in habit to *K. radicans*, but they were more slender and less hardy. The leaves were smaller and closer together on the prostrate stem.

In the leaf calcium was abundant in all cells of the water-storage region. Phosphate and inulin were not found.

In the stem potassium was abundant in the chlorenchyma, and a little was found in the pith. Calcium, inulin, and phosphate were not found.

Notonia trachycarpa Klotz.

The stem was erect, 0·8–1·0 cm. in diameter, and in most plants less than 10 cm. in height, bearing leaves fairly close together. The leaves were spathulate, 2 to 3 cm. broad, rounded at the tip and narrowing to a short base. They were light green in colour, thick and succulent. Lateral branches arose in the axils of a few leaves.

Leaf. With the exception of the colourless epidermis chlorophyll was present in all the cells of the leaf, but it was most abundant in a band at the upper surface. An irregular row of vascular bundles was present slightly nearer the lower surface.

At the narrow base there was not much calcium; it occurred in patches (Fig. 13a). In the leaf blade many cells contained a fair amount of calcium, but there were some regions where it was conspicuously absent, viz. (1) cells round each bundle, (2) a strip of cells near the upper surface, (3) some groups of cells near the lower surface (Fig. 12a).

At the leaf base phosphate was abundant, and occurred in all the cells except a few patches towards the centre, and the outermost cell-layers (Fig. 13b). The leaf blade had a little less phosphate. It was abundant in a strip of cells at the upper surface, and round each bundle; also in a few groups of cells near the lower surface (Fig. 12b). It was noticeable that calcium and phosphate are complementary in this leaf.

Small sphaerocrystals of inulin were seen in some cells round the bundles. Potassium was scattered irregularly through all tissues.

Stem. The stem exuded thick milky juice. The cortex was fairly wide, the outermost three rows of cells colourless. Chlorophyll was not evenly distri-

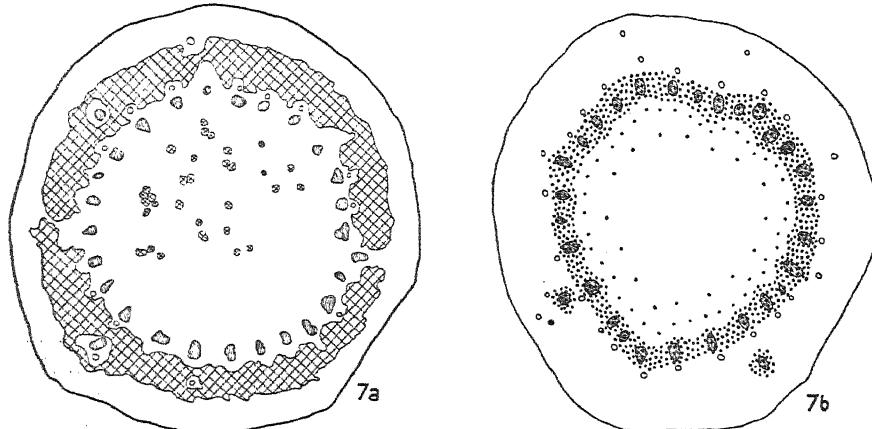


FIG. 7. *Notonia trachycarpa*: stems in transverse section. *a*, calcium in inner cortex and scattered cells of pith. *b*, phosphate (dotted) in bundle zone and (less) in outer pith.

buted throughout the rest of the cortex, but was chiefly in the outer half. The bundles were fairly close together, and the tissue between them showed very regular radial seriation. Close to each bundle, in the cortex, is a resin duct. The pith cells all contained clusters of calcium oxalate crystals, as in the pith of *K. repens* and the outer pith of *K. nerifolia*. Calcium was scarce in the pith, occurring in only a few odd cells (Fig. 7a). There was a well-defined, almost continuous zone of calcium-containing cells in the inner cortex, but the cells round leaf-trace bundles and resin ducts were devoid of calcium. The precipitate brought down by strong alcohol (i.e. calcium malate) had the same distribution as total calcium.

Phosphate was sharply localized. It was very rich in the bundle zone and over the leaf-trace bundles, but there was none round the resin ducts. There were a few smaller particles in the periphery of the pith (Fig. 7b). Here again calcium and phosphate were strikingly complementary.

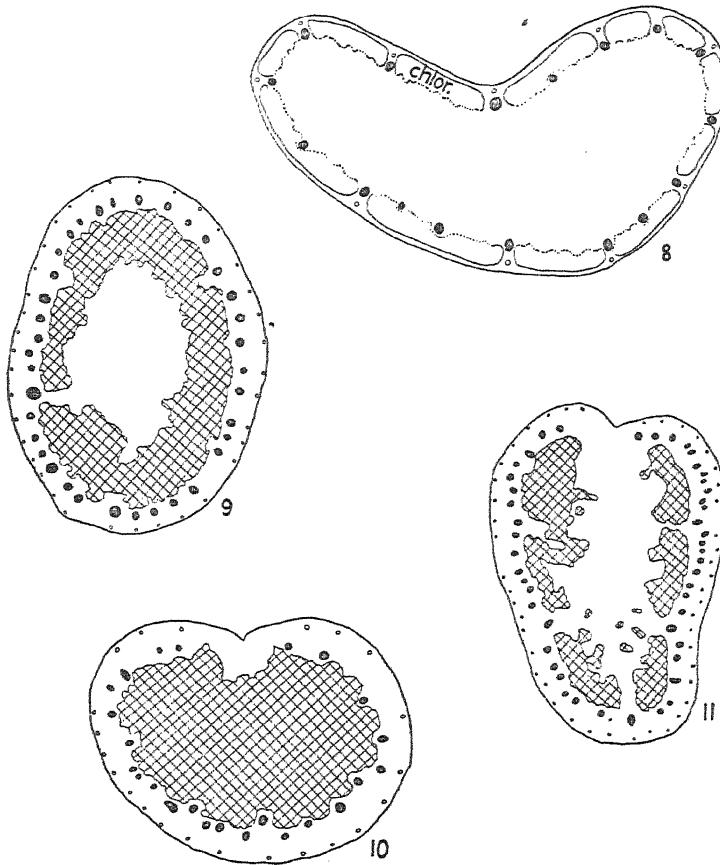
Nitrate was present, but in low concentrations. Inulin was fairly abundant between the bundles, and in the extreme periphery of the pith. The tests for potassium and sugar gave negative results.

Kleinia tomentosa Haw.

The plant available had a fairly short erect stem about 0·4 cm. in diameter. The lower 5 cm. had scars of leaf bases and a few shrivelled leaves attached to it. Leaves grew in a close spiral round the remainder of the stem, almost at right-angles to the stem, and very crowded. Each leaf was about 3 cm. long;

the basal half was cylindrical and the apical half tapered. All the leaves were entirely covered by a silvery white cottony film, easily detachable from the epidermis as a continuous fabric.

Leaf. The leaf was circular in section, with a central region of colourless



Figs. 8-11. Transverse sections of leaves. Bundles shaded; resin ducts as rings near periphery. Calcium-tissue cross hatched in 9-11. Fig. 8. *K. repens*: *chlor.*, chlorenchyma, interrupted by bundles; calcium occurs in some of the cells of the bulky water-tissue. Fig. 9. *K. aizoides*: the chlorenchyma is continuous, with sharp transition to water-tissue at the inner limit of the bundles. Fig. 10. *K. radicans*: the chlorenchyma is interrupted at the groove, and the inner margin is not as sharply marked as in *K. aizoides*. Fig. 11. *K. ficoidea*: chlorenchyma is interrupted at the groove; the transition to water-tissue is rapid in the bundle zone.

water-storage cells, and a wide assimilating zone. The bundles were in a ring in the inner part of the chlorenchyma, but two or three were found in the central zone (Fig. 14).

Calcium was found in all the cells of the leaf except the peripheral three or four layers, and a few cells round each of the central vascular bundles. The

calcium precipitate was densest in the chlorenchyma and scantier in the water-tissue. Malate had the same distribution as total calcium.

Nitrates were present in low concentration. Phosphate was absent in many leaves, and in others a few particles were precipitated in association with the vascular bundles. The amount of sugar varied. On a dull day none was found. On a sunny day there was a considerable patch of it in the water-tissue. The tests for potassium and oxalic acid gave negative results.

(The stem was not available for examination.)

Kleinia ficoides Haw.

This plant was larger than those already described. The stem was long, succulent, erect (though it needed support), and had a diameter of 1·5 cm. The leaves were arranged spirally, and were fairly close together. They were about 10 cm. long, grooved along the upper surface and flattened laterally, placed roughly at right-angles to the stem, but with an upward curve. The leaves were succulent, and contained anthocyanin which gave them a bluish colour.

Leaf. The leaf had a narrow assimilating zone, with ducts surrounded by colourless cells at regular intervals in the hypodermal region, and vascular bundles at its inner border. The bulk of the leaf consisted of water-tissue, with large, thin-walled, colourless cells. At the groove water-tissue interrupted the chlorenchyma.

Calcium was not abundant, but it was present in irregular patches in the outer water-storage cells (Fig. 11). There was slightly more calcium at the base of the leaf than at the apex. There was less calcium malate than total calcium, but the distribution was similar.

The water-tissue was fairly rich in nitrates. In some leaves no sugar was found. In others there was a little sugar right through the water-tissue, and a larger amount at the outer border of this tissue and round the vascular bundles. Very small crystals of potassium were found in the water-tissue only. Phosphate was not found.

(The stem was not available for examination).

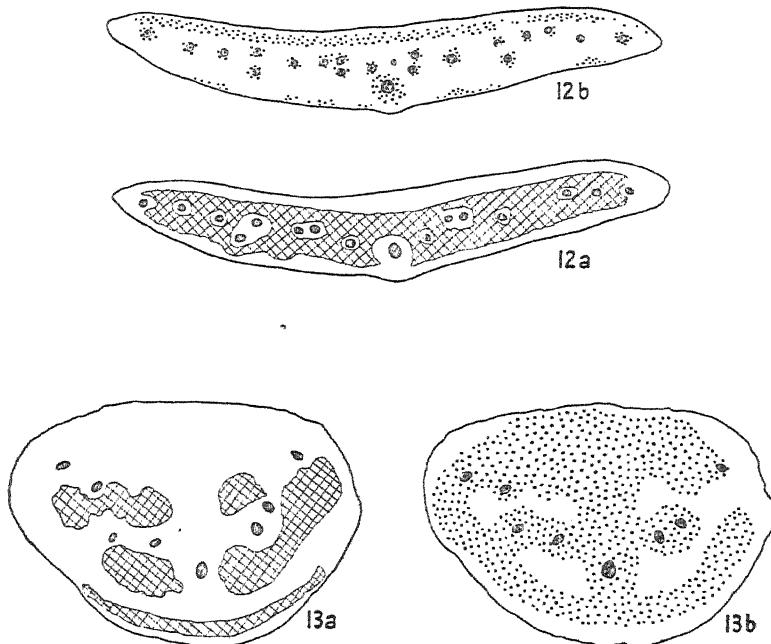
Senecio (Kleinia) stapeliaeformis Phillips.

This plant had a dark green, five-ridged stem about 1 cm. in diameter. The leaves were much reduced and finely pointed, and grew in regular rows on the ridges, close together. A lateral branch, almost equal in thickness to the parent joint, arose in the axil of one of the uppermost scale leaves.

Stem. In section the stem was star-shaped. The pith was also star-shaped, and occupied about $\frac{2}{3}$ ths of the total diameter. There was no definite line between the pith and cortex; the cortical cells were small and had abundant chloroplasts, and there were intermediate cells between them and the large, thin-walled pith cells which contained a few chloroplasts. The epidermis and two or three rows of collenchymatous cells adjacent to it were colourless. There were five main bundles, situated at the inner margin of the chlorenchyma,

one opposite each indentation; and several smaller bundles farther out in the cortex, three or four opposite each ridge. These latter were the leaf-trace bundles (Fig. 15).

Calcium was present in most of the pith cells, but some irregularly scattered



Figs. 12 and 13. *Notonia trachycarpa*: leaf. Transverse sections showing complementary distribution of calcium (cross hatched) and phosphate (dotted); bundles shaded. Fig. 12, leaf blade: *a*, calcium, *b*, phosphate. Fig. 13, base of leaf: *a*, calcium; *b*, phosphate.

cells and groups of cells had none. Practically all the cortical cells contained calcium; the epidermis, hypodermis, and two or three outermost rows of the cortex had none. The actual bundle cells were also devoid of calcium, but calcium cells approached very close to the bundles. Malate was roughly equal in amount to total calcium.

Nitrate was present in small amount. Inulin was precipitated over the vascular bundles and in the cells immediately surrounding each bundle. Some of the smaller leaf-trace bundles and strands had little or no inulin in association with them.

Sugar was present in all the cells of the pith, increasing in amount towards the outer pith, where it was abundant. There was also a large amount of sugar in the inner cortex, especially round the main bundles, though the cells of the actual bundles did not show any sugar. The outer cortex had much less sugar, and the epidermis and hypodermis none.

Tests for phosphate and potassium gave negative results.

Helianthus tuberosus.

Examination of a tuber of this plant, as an inulin-storing organ, for comparison with the succulent members of the same family, gave the following results:

A granular precipitate of inulin was produced only by the agency of absolute alcohol. It occurred in a wide band, leaving only a few peripheral rows

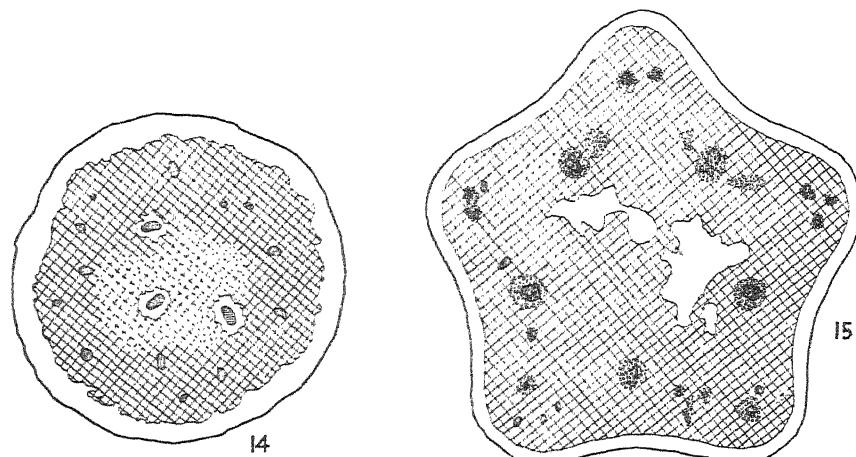


FIG. 14. Transverse section of leaf of *Kleinia tomentosa* showing scattered bundles and distribution of calcium.

FIG. 15. Transverse section of stem of *Kleinia stapeliaeformis*, showing scattered bundles, with inulin around the principal ones, and general distribution of calcium; chlorenchyma forms a broad zone including all the bundles.

of cells and a central core free. The inulin precipitate was dissolved out of the cells by water, and not reprecipitated in them by further treatment with alcohol.

There was a fair amount of phosphate in the cortex, but the bundles and meristematic cells between them had practically none. The pith was rich in phosphate except for a small central patch where there was none.

Potassium was found in all tissues, being most abundant in the pith.

Calcium was not found.

Not only the inulin but the distribution of other solutes was thus different in type from that common in the succulent stems, including the swollen rhizome of *Kleinia articulata*.

DISCUSSION

A primary object of these observations was to discover how far calcium or other solutes are consistent features of the water-tissue in succulent Compositae, and how much agreement exists between different species in respect of these physiological characters.

The facts recorded provide further illustrations of the phenomenon of

localization to which attention was called in earlier papers. This is especially well marked in the case of calcium, where, at the limits of the calciferous zone, cells with often the highest concentrations of calcium abut on cells in which none at all is precipitated. The general implications of such facts have been discussed elsewhere by one of us (Thoday 1933).

The stems of all the plants examined were succulent and the proportion of pith to cortex was high. They group themselves into those storing calcium (*a*) in both pith and (inner) cortex—*Kleinia articulata*, *K. neriiifolia* and *K. radicans*; (*b*) in the pith only—*K. aizoides*; (*c*) in the cortex only—*K. repens*. *Notonia trachycarpa* may be regarded as intermediate between (*a*) and (*c*), since only scattered cells of the pith contain calcium in their sap. The case of (*d*) *K. stapeliaeformis* is different in that this species is a stem succulent with minute leaves only, the bundles are scattered and calcium storage more general.

In leaves of centric type, in which the bundles are arranged in a definite zone near the outside, separating more or less clearly outer chlorenchyma from a pale or colourless water-tissue, calcium is present in the latter only. In *K. repens*, not every cell contains it; in *K. ficoides*, *K. aizoides*, and *K. radicans* it is present in ascending order of amount. In the flat leaves of *K. neriiifolia* and *Notonia trachycarpa* calcium still avoids the bundles. The cylindrical leaves of *K. tomentosa*, with scattered bundles, may be compared with the stem-succulent *K. stapeliaeformis*, for calcium is more generally distributed, though absent round the principal bundles.

It is noteworthy that in the few stems in which calcium is not accumulated by the cells of the pith, calcium oxalate crystals are found. They are general in the pith of *K. repens*, and in that of *Notonia trachycarpa* except for the few cells containing calcium in solution, and are found also in the otherwise calcium-free outer pith in *K. neriiifolia*. This does not, however, apply to leaves.

In these Compositae no case has come to light comparable with *Mesembrianthemum violaceum* (Thoday and Evans, 1932) in which oxalate formation and calcium malate accumulation occur in the same cell; though the presence in the calcium oxalate cells of malic acid balanced by some other base is not excluded by the evidence available.

The occurrence of phosphate shows considerable variation in individual species at different times, and in some has not been observed. Usually, as in *K. articulata* and *K. neriiifolia*, it is confined when present to the bundle zone, and its distribution is complementary to that of calcium, as it commonly is in the stem of *K. articulata*. The leaves of *Notonia trachycarpa* and *K. neriiifolia* provide especially noteworthy examples of complementary distribution.

With increased accumulation, however, phosphate may encroach on calciferous regions, spreading, as in stems of *K. articulata* which have received additional phosphate, from the bundle zone into the outer pith, or the cortex, or both. In stems of *K. repens* and *K. radicans* it was sometimes present in

all the cells of the pith. The presence of calcium salts in the sap (or the metabolic make-up of which calcium accumulation is one expression) is therefore not an absolute barrier to phosphate accumulation, although non-calciferous cells accumulate it more readily. There is evidence that oxalate-forming cells accumulate phosphate more readily than malate formers.

Localization of soluble carbohydrates has also been demonstrated. Inulin is usually confined, like phosphate, to the neighbourhood of vascular bundles. Here again, however, in the rhizome of *K. articulata*, if it becomes more abundant it spreads to other tissues, including calciferous pith.

It might at first sight be thought that inulin is stored in the cells nearest to the bundles because these are the first to be reached by carbohydrates via the bundles. The case of *Kleinia stapeliaeformis*, however, cannot readily be interpreted in this way. The leaves are minute, the photosynthetic tissue covers the surface of the stem and the movement of carbohydrate must be rather inwards toward the bundles than along them and outwards from them. Moreover, reducing sugar has been found generally distributed in the parenchyma of the stem in this plant.

By analogy with starch-sugar equilibria, differences in 'critical concentration' for inulin formation are conceivable. Localization might still be explained in part by limitation of supply; but considerable differences of critical concentration would be needed at the normal boundary to account for the uniformity, notwithstanding considerable variations in amount, of the normal distribution.

The difference between rhizome and aerial stem of *K. articulata* can be interpreted similarly as depending on a lower critical concentration for inulin formation in the former. The segregation of reducing sugar and inulin in older stems of *K. neriifolia* seems to call for a corresponding interpretation: inulin is found in the bundle zone only, while reducing sugar is abundant in the pith; but the accumulation of sugar in the first place remains unexplained, for even the green cortex shows none; starch is formed in the chloroplasts in the outer cortex.

Other cases of localization of reducing sugar have been noted. Such facts, however, as its presence in the pith of young stems of *K. articulata* and its rarer presence only in the bundle zone and adjoining cortex in older stems, its absence from some species and localization in others, are phenomena which require more intensive investigation in relation to external conditions, seasonal changes, &c. In leaves of *K. tomentosa* and *K. ficoidea*, sugar was found on sunny but not on dull days. It is not unlikely, too, that inulin would have been found in species which gave negative results if older material had been available.

Making full allowance for such limitations in the data, it may still be concluded that these succulent organs exhibit very generally a physiological differentiation of their parenchymatous tissues into regions or zones sharply separable from one another by their different behaviour in relation to calcium,

inulin, phosphate, and sometimes other solutes. In relation to calcium the difference appears to be qualitative. In other cases it is possible to regard the differences as of degree only.

SUMMARY

1. Inulin occurs in old stems of *Kleinia articulata* strictly confined to the parenchyma of the bundle zone. In the rhizome the inulin zone is wider and may extend far into the pith, overlapping calcium malate extensively.

2. Reducing sugar commonly occurs in young leafy stems of *K. articulata*, always confined to the pith, as far as can be determined by precipitation *in situ* with Fehling's solution. In old stems it is detected, if at all, only in part of the bundle zone and inner cortex, not in the pith.

More chloride was found in the pith than in the cortex.

3. Cuttings of *K. articulata* gave no indications of mobilization of calcium malate in the neighbourhood of sprouting roots or shoots. Starvation led to injection by sap escaping first from the pith, by which time the calcium malate had largely disappeared and the sap showed a high pH of 8.0–8.5.

4. In the stem of *K. nerifolia* an outer zone of the pith contains no sap calcium, but crystals of calcium oxalate in each cell; phosphate distribution normally extends into this zone. In all but young leafy joints, inulin was abundant in the bundle zone. In the leaf, calcium and phosphate occur, with complementary distribution.

5. In the stems of several other species the distribution of calcium showed variations on the *articulata-nerifolia* pattern; and in leaves with bundles round the periphery of a wide central water-tissue, calcium was confined to the latter.

6. In the flat leaf of *Notonia trachycarpa*, calcium and phosphate were complementary in their distribution.

7. The leaf of *K. tomentosa* and the leafless stem of *K. stapeliaeformis*, both with scattered bundles, show more general distribution of calcium, which, however, avoids the larger bundles. In the latter species, inulin occurs closely associated with the bundles.

8. In the slender stem of *Othonna carnosa*, no calcium was found.

9. The inulin in these succulent Compositae contrasts with that in the tuber of *Helianthus tuberosus* by the readiness with which it is precipitated by various concentrations of aqueous alcohol. Strong alcohol produces immediately a granular precipitate; the weaker the alcohol the larger and fewer the sphaerocrystals formed.

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Studies in Tropical Fruits

IV. Methods in the Investigation of Respiration with Special Reference to the Banana

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With six Figures in the Text

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I. INTRODUCTION

IN earlier papers in this series emphasis has been laid on the need for conducting respiration studies on as comprehensive a basis as possible, measurements of the internal concentrations of carbon dioxide and of oxygen and also of the amounts of carbon dioxide contained in the tissues being considered essential adjuncts to the measurement of carbon dioxide liberated at the surface of the fruit, especially where changes are taking place in this liberation rate. Methods have now been devised for use with fruits without an internal cavity, and in particular the banana, whereby the required data may be obtained either from consecutive observations on the same fruit or on closely comparable fruits. In addition, in view of the importance which tissue resistance to the movement of gases may assume in respiration, manometric methods have been employed to determine directly changes in pressure within the fruit. The readjustments in the concentrations of

carbon dioxide and oxygen within the fruit which take place in response to changes in the external atmosphere have also been examined by appropriate methods as an approach to the investigation of gas storage phenomena. Some preliminary measurements of the temperatures of the flesh during ripening have also been made. In so far as the porosity of the tissues of the skin and flesh may be affected by transpiration and by the changing water-relations within the fruit during ripening, measurements of rates of water-loss during ripening have been undertaken as an integral part of these studies (Leonard, 1939). It may also be mentioned that biochemical studies, based on lots of fruit comparable to those used in respiration experiments, are also being undertaken.

The arrangements of apparatus and equipment which have been used in these investigations of respiration are described below.

II. MEASUREMENT OF RESPIRATION RATES

In their investigation of respiration rates the writers have used the system in which the organ under examination is held in a respiration chamber and the carbon dioxide liberated is measured by a continuous draw-through and absorption system. Both in standard works and general literature the details of setting up and operating this type of apparatus are usually treated very briefly, the underlying assumption being that the records obtained are a fair representation of the normal respiration of the plant organ under consideration, in air. As a result of (a) the high respiration rates typical of bananas during ripening, particularly at tropical temperatures, (b) the very marked sensitivity of the fruit to small temperature changes especially at certain critical stages, e.g. the climacteric, and (c) the very rapid rate at which changes in the respiration rate occur, the writers have been forced to consider critically the working of this type of respiration apparatus.

The factors operating inside the respiration chamber which are usually regarded as fully and independently controllable, are (a) the gaseous composition of the air, (b) its temperature, (c) the rate at which air is drawn through, and (d) its humidity. In practice, however, it has been found that the system is considerably more complicated, the factors being neither completely nor independently controllable. Some explanation of these points may, with advantage, be given here.

Various workers (Spoehr and McGee 1924, Willaman and Beaumont 1928), have commented on the effect of even slight accumulations of carbon dioxide within the respiration chamber. By reducing the amount of 'dead' air in the respiration chamber and by using a high rate of flow this defect of a large respiration chamber can be offset but other difficulties are introduced, e.g. the absorption of the carbon dioxide. In practice it has been found advantageous to use a respiration chamber which is closely adapted to the size and shape of the fruit under investigation. For individual banana fruits, the writers have found that cylindrical glass tubes of different diameters to accommodate different grades of fruit are suitable, Fig. 1.

When a banana is held in such a tube, the heat generated by the fruit sensibly alters the temperature of the air within the respiration chamber. Furthermore, since the rate of production of heat during ripening shows a considerable rise, it follows that, although the temperature of the incoming air may be maintained constant, the actual values within the chamber may vary during the course of the experiment. This is illustrated in Fig. 2 which

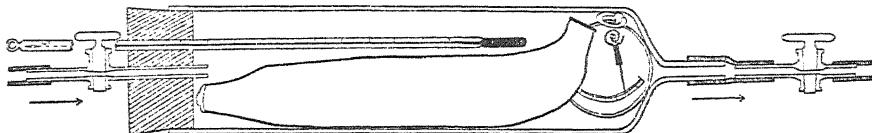


FIG. 1. Respiration chamber, with fruit, hygrometer, and thermometer in position.

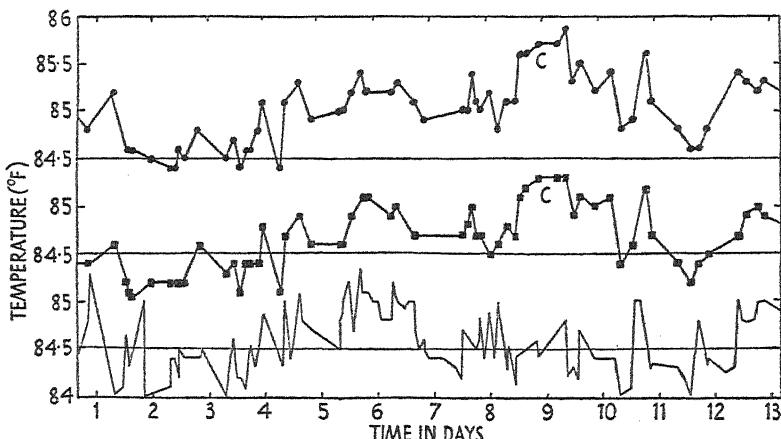


FIG. 2. Record of temperatures observed inside banana respiration chambers maintained at high (upper graph) and low (middle graph) relative humidities during an experiment at 84.5°–85°F. A comparable record for the storage room is shown (bottom graph). C, indicates the point at which the peak of the respiration climacteric was reached.

shows the temperature records obtained in respiration experiments at two humidities in respiration chambers within the same room.

Again, in so far as transpiration is productive of a cooling effect on the fruit, the rate of transpiration, as influenced by (i) the relative humidity of the air supplied and (ii) the rate of draw-through, is also a factor modifying the temperature of the air surrounding the plant organ. This is illustrated by the difference between the upper and middle records, Fig. 2. As the rate of loss of water in transpiration is about three times that of the carbon dioxide of respiration even in a saturated atmosphere only partial control of relative humidity is possible; in point of fact a gradation of increasing relative humidity from the inlet to the outlet side of the respiration chamber can be demonstrated.

In addition to the points mentioned above, it is to be noted that in any 'draw-through' system using suction alone, as contrasted with a 'push-pull' system, the fruit is unavoidably subjected to a slight reduction in pressure.

The details of the system used may now be briefly described. The data for individual experiments will be tabulated in subsequent papers. The apparatus consists of the following units in sequence, (1) an inlet tube supplying fresh air, (2) soda-lime absorption tower with Sofnlolite indicating layer, (3) dilute baryta with phenolphthalein guard bottle, (4) wash bottle containing saturated salt solution for controlling relative humidity, (5) respiration chamber, Fig. 1, (6) carbon dioxide sampling bottle (inserted periodically), (7) Pettenkoffer absorption tube, (8) baryta indicator bottle as in (3), (9) capillary gas flowmeter with screw-clip control, (10) mercury snifting valve leading to suction line with needle-valve control.

The following annotations on the functions of the several integral parts may be of use to other workers.

The use of different saturated salt solutions for the control of humidity has been advocated by various workers. The limitation of control possible in this particular arrangement of apparatus has been indicated.

The respiration chambers used are $2\frac{1}{2}$ in. or $2\frac{3}{4}$ in. diameter and 10 in. long having capacities of 750 or 900 c.c., and accommodating bananas of 130–160–190 gm. weight, the chief limitation being the variations in curvature among the individual fingers. The wide end of the tube is closed with a rubber bung (Fig. 1). A small paper hygrometer is placed in each respiration chamber at the suction end.

Immediately following the respiration chamber a small gas-sampling bottle, about 40 c.c. capacity, is inserted and periodically withdrawn for analysis of the carbon dioxide concentration, thus affording an indication of the composition of the respiration chamber atmosphere. Pettenkoffer tubes containing 50 c.c. of N/10 baryta and barium chloride are attached and withdrawn for titration with N/10 hydrochloric acid at intervals as required.

The capillary flowmeter is of the type described by Hardy (1931). The limitation to the rate of draw-through in this lay-out of apparatus is that imposed by the absorption capacity of the Pettenkoffer tube; it was found to be about 200 c.c. per minute. At this rate, the carbon dioxide concentration within the respiration chamber, when a fruit weighing 150 gm. was liberating carbon dioxide at the peak value of the climacteric at 85° F.—about 300 mg/kg/hr—could be maintained at not more than 0·4 per cent., with a maximum loss of carbon dioxide, due to non-absorption, amounting to 1·5 per cent.; this loss was determined by attaching a second Pettenkoffer tube in series with the first. With a fruit of about 150 c.c. volume a rate of 200 c.c. per minute gives a change of air about once every three minutes. The reduction in pressure within the chamber below that of the outside atmosphere amounts to about 5 to 10 cm. of Brodie fluid.

In conjunction with the measurement of respiration rate, arrangements were made for analysing the concentrations of the gases within the fruit contained in the respiration chamber. These are described in the next section.

III. MEASUREMENT OF INTERNAL GAS CONCENTRATIONS

Whereas in large fruits with large internal cavities such as the papaw, or in those with open storage parenchyma, such as the water-melon, no difficulty is encountered in withdrawing samples of 10 c.c. for determination of carbon dioxide and oxygen concentrations (Wardlaw and Leonard, 1936), the withdrawal under reduced pressure of even small samples from the interior of small compact fruits, such as the banana or mango, may prove difficult in itself and yield results which are not truly representative of the normal internal atmosphere. The writers, however, have devised a method by which samples can be withdrawn at intervals from the same banana or mango fruit throughout the course of ripening. As a result the trends of the internal concentrations of carbon dioxide and oxygen can be determined and used in conjunction with other respiration data and observations on maturation processes.

The method is as follows. Using a cork-borer, sterilized with 0·2 per cent. mercuric chloride solution, a plug of tissue is withdrawn from the fruit and a gas sampling tube similarly sterilized, consisting of a 5 c.c. bulb with three-way capillary stop-cock, is inserted, and sealed with a rubber washer and vaseline, Fig. 3 (i). Only in rare instances have fruits had to be discarded before final senescence on account of fungal rotting consequent on the above operation. Samples for analysis are withdrawn by attaching one arm of the 3-way stop-cock to the portable Haldane gas analysis apparatus. The latter is previously manipulated so as to contain a known volume of nitrogen (approximately 7·5 c.c.) which is shunted into the caustic potash pipette. The mercury column is then raised so as to reach the free arm of the 3-way stop-cock, thus displacing air from the lead-in tube. The stop-cock is now turned so as to connect the 5 c.c. bulb with the lead-in tube, and the mercury lowered so that a gas sample of approximately 2 c.c. is drawn into the gas burette of the Haldane apparatus. The 3-way stop-cock of the gas-sampling tube is then closed.

The nitrogen is now brought back from the caustic pipette to the gas burette and the analysis of carbon dioxide and oxygen is carried as in the dilution method described in 'Methods of Gas Analysis' (Haldane, 1920).

The principle underlying this method of determining internal gas concentrations is that the atmosphere originally present in the bulb of the sampling tube will come into equilibrium with the internal atmosphere of the fruit. Several experiments tend to show that the assumption involved is correct. Thus when consecutive samples are withdrawn at short intervals from a green fruit, whose resistance to gaseous movement is known to be low (Wardlaw and Leonard, 1936), the concentrations of both carbon dioxide and oxygen observed show a high degree of consistency. On the other hand, in a senescent fruit, whose resistance to gaseous movement is high, the effect of withdrawing consecutive samples is to produce a progressive increase and decrease in the carbon dioxide and oxygen concentrations respectively. Again, in manometric

studies (see section IV) the trend of internal pneumatic pressure obtained during ripening is in close agreement with the trend of the curve for carbon dioxide plus oxygen (Wardlaw and Leonard, 1938). It has also been observed that considerably greater difficulty is experienced in withdrawing samples from senescent than from green fruit.

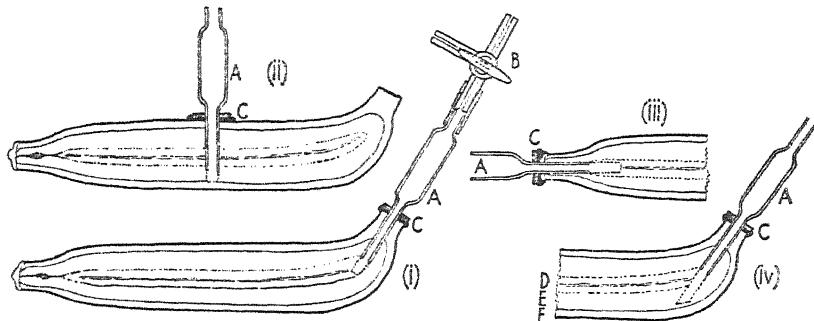


FIG. 3. Diagrams illustrating the several ways in which the gas-sampling tube may be inserted into a banana: (i) placental region or inner flesh, basal end; (ii) outer flesh, half-way along the fruit; (iii) placental region, apical end; (iv) basal end, with both inner and outer flesh sampled. A, 5-c.c. gas-sampling tube; B, 3-way capillary stop-cock; C, rubber washer and film of vaseline; D, basal end of inner flesh; E, outer flesh; F, skin.

As will be shown in the sequel to this paper, the internal concentration of carbon dioxide bears a direct relation to the quantity of carbon dioxide contained in the tissue ('tissue content') which is high. On withdrawing successive small samples, therefore, the carbon dioxide of the internal atmosphere is readily replaced, so that the original equilibrium between the internal gaseous carbon dioxide and that in solution (i.e. the tissue content) is regained.

The internal oxygen concentration is dependent on several factors, including (i) the resistance which the tissues offer to gaseous movement, (ii) the gradient of concentration between the external atmosphere and the interior, and (iii) the extent of utilization of the oxygen as it passes across the tissues to the region of the sampling tube. As between any two closely consecutive samples, the tissue resistance, which varies according to the maturity of the particular fruit, will be the most important of the three factors enumerated. In a green fruit (with low resistance, i.e. high porosity) the oxygen concentration will remain constant or may tend to rise slightly, whereas in a senescent fruit, where the tissue resistance is known to be high, the oxygen concentration will tend to fall with successive samples, while the carbon dioxide, which is present in large quantities in the tissue, will be drawn out, thereby increasing its percentage concentration in the sample.

It is realized that the above facts and observations do not in themselves constitute an exact proof that the method of sampling adopted yields precisely accurate data on the composition of the internal atmosphere in a small, solid fruit. Nevertheless the results obtained have given well-marked and well-

substantiated trends, these being generally comparable with those obtained for large fruits as the papaw, and results such as can be closely correlated with other respiration data and ripening observations in the banana as a whole.

Certain limitations are inherent in this method of sampling. In the first instance a certain but variable amount of wounding is unavoidable, and secondly, the insertion of the sampling tube with its 5 c.c. bulb must cause an initial alteration in the composition of the internal atmosphere. The collective experience from many experiments, however, indicates that, in fruit held at 85° F., both these effects almost completely disappear within twenty-four hours. It is thus impossible to obtain reliable figures for the composition of the internal atmosphere immediately after separating the 'finger' from the bunch, a period known to be of special interest in respiration and transpiration studies. By inserting the sampling tube in fruits still attached, however, this can be overcome.

The manner in which the sampling tube is inserted and its position have also been found to be of considerable importance. This is explicable by the fact that ripening takes place regionally, the major changes in the internal concentrations of carbon dioxide and oxygen for the several organographic regions being definitely separated in time. The evidence available indicates that the sample withdrawn is restricted to a relatively small region in proximity to the end of the sampling tube, including the small cylindrical cavity left by the withdrawal of the plug of tissue. Uniformity in the position of the sampling tube is therefore essential where close comparisons are being made between fruits.

Fig. 3 indicates how variations in the insertion of the tube may, in terms of regional ripening, yield considerably different data on internal gas concentration. Fig. 3 (iv) is an instance in which the atmosphere of both the placental region, in which ripening may have begun, and the outer flesh, in which ripening has not yet been initiated, will contribute to the samples withdrawn. While it is a fairly simple operation to localize exactly the sampling tube, occasions may occur in which the incision of the cork-borer may have been more extensive than was intended. During the final anatomical inspection of the fruit, however, observation of the insertion of the sampling tube affords information which may be used in conjunction with the records obtained. In Fig. 3 (ii, iii) other positions in which sampling tubes may be inserted in the investigation of regional distribution of gas concentrations are also shown.

In practice, in order that sufficient data may be obtained to indicate major changes within the fruit it has been found sufficient, for bananas at 85° F., to sample twice a day during the preclimacteric phase, four to five times a day during the climacteric, and again twice a day subsequently. Such a programme of sampling does not appear to be accompanied by abnormal effects on the fruit.

In order that changes in respiration rate may be precisely correlated with changes in the composition of the internal atmosphere, the arrangement of

apparatus illustrated in Fig. 4 has been devised. The rubber bung which closes the wide end of the respiration chamber is provided with a 5 c.c. internal-gas-sampling tube and 3-way stop-cock as well as accommodating the inlet tube. The banana is bored at the apical end and the sampling tube inserted,

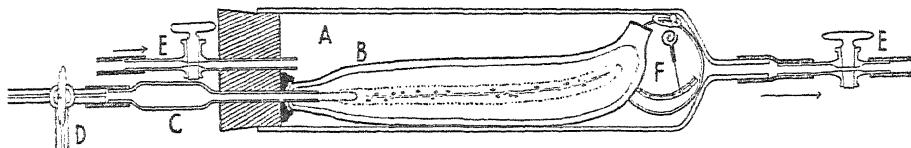


FIG. 4. Arrangement of apparatus to permit of both measurement of the respiration rate and of analysis of the internal atmosphere of a banana. A, respiration chamber; B, banana, in longitudinal section, showing the cavity made by the cork-borer; C, sampling tube, with D, 3-way stop-cock; E, 2-way stop-cock; F, paper hygrometer.

sealed with vaseline. When gas samples are to be withdrawn for analysis, the respiration chamber is temporarily detached from the respiration set, the stop-cocks at either end being closed, and a sample of gas withdrawn as described above.

Lastly, it may be noted that little or no difficulty has been experienced in continuing sampling until the final stages of senescence when the flesh becomes semi-liquid. It may also be mentioned that this method of sampling has been used successfully with the mango, a solid fruit with a large central stone, and could be used to advantage with the smaller citrus fruits; the larger citrus fruits, such as the grape fruit, and fruits of open texture such as the egg-plant, can be sampled by withdrawing 8-10 c.c. samples using a simple sampling tube—i.e. without 5 c.c. bulb.

IV. MEASUREMENT OF PNEUMATIC PRESSURES

In an earlier paper (Wardlaw and Leonard, 1938) it was shown that by attaching manometers to several different tropical fruits, considerable departures from the normal atmospheric pressure are readily demonstrated during development and ripening. In the papaw these could be closely correlated with data, obtained from the same fruits, of the internal concentrations of carbon dioxide and oxygen, and further with observations on developmental and ripening changes. The manometer record, which gives the differences between the sum of the partial pressures of the gases present in the external and internal atmospheres, is of considerable value in the study of respiration and transpiration.

In the application of this method to small, compact fruits such as the banana or mango, precautions must be taken to ensure that the manometer is sufficiently sensitive, and closely coupled to the fruit so as to avoid a disproportionately large dead-air space between the manometer fluid and the internal atmosphere of the fruit. Capillary tubing of 1.5 to 2.0 mm. bore, and Brodie solution were used. As noted in Section III above, the insertion of

the sampling tube involves wounding and disturbance of the normal internal atmosphere. The manometer records obtained indicate that these effects disappear within 24 hours.

V. CARBON DIOXIDE CONTENT OF TISSUE

In earlier papers (Wardlaw and Leonard, 1936, 1938) the writers have shown the necessity of obtaining data on the carbon dioxide of the tissues. The desirability of obtaining corresponding data on the oxygen content of tissues is also evident, but so far no satisfactory method has been worked out. By the term 'tissue content of carbon dioxide' is understood the total carbon dioxide in a tissue which may be present (i) as gaseous carbon dioxide in the intercellular air spaces, (ii) as carbon dioxide in solution in cell wall and protoplasm, retained under physical or physiological conditions as yet unexplored, and (iii) as carbon dioxide in a combined form.

The determination of these several components obviously calls for a diversification of method and extended investigations. As a preliminary approach to the problem, the writers have adapted the method of Willaman and Brown (1930), who investigated the carbon dioxide content of twigs, to fruits. These authors have contributed an interesting bibliography on the question of dissolved carbon dioxide in plant tissues.

Method and Apparatus.

The method consists essentially of (i) a rapid killing of tissues by immersion in alcohol, (ii) the driving off of carbon dioxide by boiling and irrigating with carbon dioxide-free air, and (iii) the absorption of the carbon dioxide driven off.

The apparatus which has proved satisfactory for banana tissues consists of the following parts in sequence: (1) an inlet tube supplying fresh air, (2) Wallace wash bottle with strong caustic soda, (3) dilute baryta with phenolphthalein guard bottle, (4) a distillation flask (500 c.c.) fitted with a side-arm gas lead-in tube, (5) Davis double-surface condenser, (6) alcohol trap flask, (7) Pettenkoffer tube, (8) guard bottle as in (3), (9) mercury snifting valve leading to suction line with needle-valve control. Fig. 5 shows the central feature of this apparatus. Where possible ground-glass joints were used; elsewhere heavy rubber tubing thoroughly vaselined was employed for joining glass tubing, adjacent ends being coupled as closely as possible to minimize leakage of carbon dioxide.

The distillation flasks which are provided with ground glass stoppers and contain 150 c.c. of alcohol, are weighed. The tissue to be examined is prepared as quickly as possible, dropped into the alcohol and the flask and contents weighed. The flask is then placed in position in a water bath under the condenser. In the meantime the Pettenkoffer tube, supplied with 50 c.c. of N/20 baryta and barium chloride has been placed in position. Suction is now begun at a suitably slow rate, this being subsequently increased to 200–250 c.c.

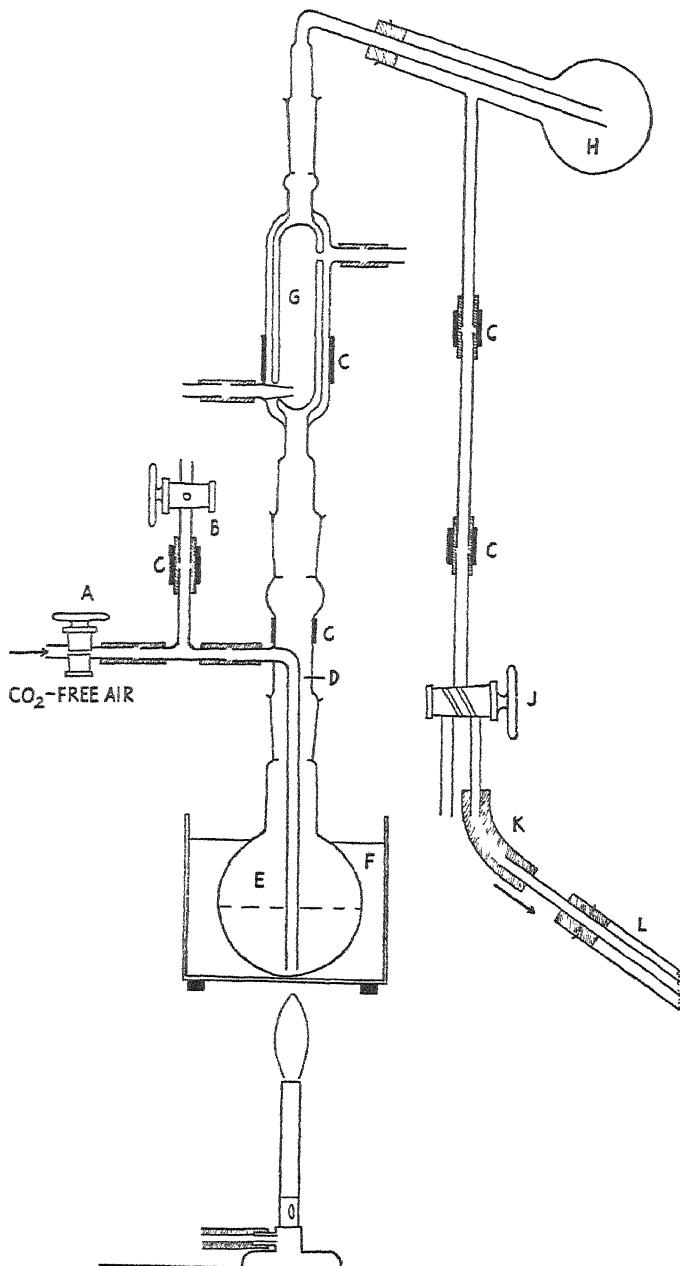


FIG. 5. Apparatus used for determining the carbon dioxide content of the tissue. A, stop-cock permitting access of carbon dioxide-free air; B, stop-cock on side-arm for emergency adjustments, &c.; C, supporting clamps; D, side-arm gas lead-in tube; E, 500-c.c. distillation flask, containing 150 c.c. alcohol and tissue; F, water bath; G, condenser; H, alcohol-trap flask; I, 2-way stop-cock; K, heavy rubber tubing; L, Pettenkoffer tube.

per minute. The water bath is heated to boiling point. If necessary a second Pettenkoffer tube replaces the first after a suitable interval. The baryta is titrated against N/20 hydrochloric acid using phenolphthalein as indicator.

As the apparatus initially contains an atmosphere of 800 c.c. of air (with about 0.0390 percent. CO₂) and the alcohol used also contains a small quantity of dissolved carbon dioxide, a blank estimation using 150 c.c. of alcohol must be carried out.

The tissue content of carbon dioxide is expressed as mg. of carbon dioxide per kg. of tissue. Such determinations of tissue content of carbon dioxide are made on fruits whose internal carbon dioxide and oxygen concentrations had been followed up to the time of sampling. The tissue in alcohol was finally stored for subsequent biochemical analysis.

When a blank estimation is made by means of a series of samples of carbon dioxide liberated from 150 c.c. alcohol, taken at intervals in the course of four hours or more, it is found that the data yield an asymptotic curve, the major quantity coming off as the alcohol is brought to the boil during the first 40–60 minutes. Thereafter liberation proceeds at a steady, slow rate which, in some instances has been followed for eight hours. If the carbon dioxide in the dead air of the apparatus is considered to be present at a concentration of 0.03 per cent., and if a direct titration of the alcohol itself is made (by adding 50 c.c. of N/20 baryta and phenolphthalein and titrating with N/20 hydrochloric acid) the total amount of carbon dioxide thereby determined is slightly less than that observed when the blank estimation of 150 c.c. of alcohol is made by the boiling and draw-through method over a period of four hours. Various factors, including higher concentrations of carbon dioxide in the laboratory (and therefore initially present in the apparatus), and the passage of acid gases into the system through the rubber joints, may be held partly accountable for the differences observed.

In a blank estimation of 150 c.c. of alcohol the quantities of carbon dioxide involved (and in consequence the titration values obtained) are very small. To explore more fully the type of curve obtained when carbon dioxide is being boiled out of alcohol under the conditions described, 150 c.c. of alcohol was charged with commercial carbon dioxide from a gas cylinder, and the estimation of the gas carried out. The results obtained are shown in Fig. 6, where it will be observed that, after the initial large amounts of carbon dioxide have been liberated, the trend of the curve is almost exactly parallel with that obtained in the blank estimation.

Fig. 6 also shows the curves obtained during the determination of the carbon dioxide content of the skin and flesh of a green, unripe banana. Again it will be seen that the major part of the carbon dioxide was discharged during the first hour, and that the trends of the latter portions of the curves are closely comparable with that of the blank and that in which the alcohol had been charged with carbon dioxide. For tissues yielding curves of this type it is evident that estimations may be based on an extraction period of two to four

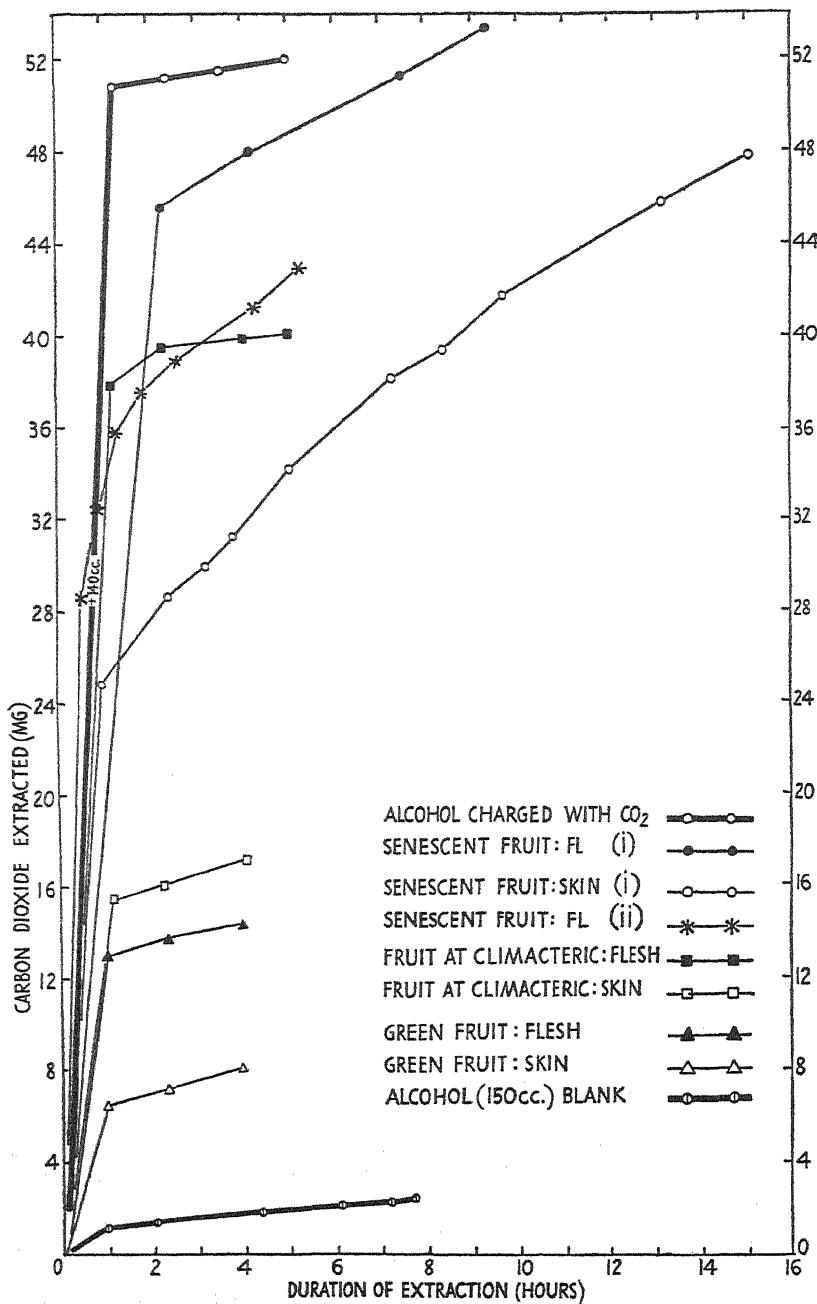


FIG. 6. Liberation of carbon dioxide from alcohol and from the skin and flesh of green, ripening (at climacteric stage), and senescent banana fruits. Asymptotic curves are obtained from the normal alcohol blank, from alcohol charged with carbon dioxide and from green and ripening fruits. Both the skin and flesh of senescent fruits, by contrast, continue to yield considerable quantities of carbon dioxide even after an extraction period of four hours.

hours, the blank estimation curve being used to give the necessary correction. Willaman and Brown (1930) state that an extraction period of two hours was suitable for the estimation of carbon dioxide in apple and cherry twigs.

Whilst the carbon dioxide content of the skin tissue of an individual green banana fruit may be small, in comparison with the blank, the close parallelism between the latter parts of the curves would suggest that the results obtained are nevertheless dependable; the larger the quantity of tissue used, the smaller will be the proportion of the value of the blank to be subtracted, and the greater the accuracy. In certain investigations, as in a regional survey of the tissue content of carbon dioxide of a single fruit, only small quantities of tissue may be available for each estimation. In these instances it is advantageous to use a smaller apparatus and a smaller quantity of alcohol; there are, however, obvious limitations to the extent of such reduction.

The above method has been found uniformly applicable to the skin and flesh of banana fruits until the beginning of final senescence. In the senescent fruit, on the other hand, the curve obtained is somewhat different. The initial rapid discharge of carbon dioxide from the boiling alcohol takes place as in the other instances cited, but during the subsequent period the curve obtained is not quite in agreement with those already considered, being considerably steeper and showing only a slight falling off, even after nine to fifteen hours. For a complete investigation of the sources of carbon dioxide in such senescent fruit it is evident that the significance of the type of carbon dioxide liberation curve obtained will require critical analysis, and that the method of estimation may call for a modification.

Some further observations on the use of this method may also be noted.

Agreement between replicates.

The closeness of agreement between replicates was tested as follows. The soft flesh of three senescent fruits was thoroughly pulped, mixed, divided into three portions, and the estimation of carbon dioxide content carried out as described, the time to bring to the boil, the rate of aeration, and the duration of the estimation being the same in each case. The variation was of the order of 6 per cent. about the mean.

Loss of carbon dioxide during sampling.

Since any wounding of a tissue causes a rapid discharge of carbon dioxide, some loss in the carbon dioxide content of a tissue is inevitable in the method adopted. To a large extent such losses could be minimized by removing plugs from skin to skin by means of a cork-borer (Magness, 1920), but regional analysis would be thereby precluded, and the proportion of flesh to skin would not be the same as that in a whole fruit.

The extent of losses in carbon dioxide sustained during the preparation of samples was investigated by withdrawing samples of flesh thoroughly mixed to a pulp and placing them in flasks after intervals of one, three, and six minutes

from the commencement of cutting open the fruits. The tissue contents of carbon dioxide were as follows:

After 1 minute	289·4	mg. per kg.
" 3 "	269·7	"
" 6 "	262·0	"
Mean value	273·7	"

There is a slight decrease in carbon dioxide content with time, but the total variability is again seen to be of the order of 6 per cent. about the mean. Inasmuch as the normal time required for the preparation of an individual sample is less than one minute, the variability between samples is probably of greater importance than the loss of carbon dioxide from individual samples.

Effect of delay in estimation.

Where a large number of samples is being handled it may be impracticable to commence the estimation immediately after sampling. The effect of a delay of twenty-four hours as compared with an immediate estimation was tested but no marked difference in the carbon dioxide content of comparable samples was observed.

Carbon dioxide in combined form.

After an initial extraction of banana tissue with alcohol and estimation of carbon dioxide, 50 c.c. of alcohol made acid with citric acid (0·1%) was added, and further estimations made. The latter results showed no departure from the asymptotic curve already established. It therefore appears that if there is any carbon dioxide present in a combined form, it is liberated as a result of boiling with the acids already present in the tissue. Accordingly the use of acid alcohol has not been adopted in these estimations.

Alcohol used for extraction.

The alcohol used for extractions was prepared by distilling crude molasses-spirit over caustic soda. The resulting distillate contains approximately 80 per cent. ethyl alcohol, and has a very low titratable acid content.

VI. OBSERVATIONS ON GRADIENTS OF GASEOUS CONCENTRATION

It has been shown for the papaw and other fruits (Wardlaw, 1936) that, when a fruit is removed from one gaseous medium to another, readjustments of the concentrations of the internal gases may take place with considerable rapidity. In such instances data on gradients of gaseous concentration are essential for the correct interpretation of the changing rates of respiration observed. Different fruits of the same variety, and the same fruit at different stages in its development and maturation, may show marked differences in the resistance which the tissues offer to the movement of gases. Here again, among the complex of factors involved, gradients of gaseous concentration may be important. The writers hold that a knowledge of this factor is essential to the rational development of gas-storage technique.

Apparatus for observation of gradients of gaseous concentration as affecting large fruits such as the papaw has already been described (Wardlaw, 1936). For individual banana fruits the following arrangements have been found useful. The fruit, provided with a gas-sampling tube and 3-way stop-cock, is placed in a respiration chamber as shown in Fig. 4. The chamber is provided with an inlet tube and stop-cock, and a 3-way stop-cock is attached at the narrow end; this may be coupled periodically to the Haldane gas analysis apparatus or to a sensitive manometer during the intervals between sampling, as required. A thermometer and paper hygrometer indicate the temperature and humidity of the chamber as in Fig. 1. The fruit may be held in a closed system for observations of the changing concentrations of carbon dioxide and oxygen within the chamber and fruit as respiration proceeds and carbon dioxide accumulates, or other gases, e.g. nitrogen, may be introduced and appropriate analyses undertaken.

It has already been seen that when the atmosphere within the chamber is at a high relative humidity, its temperature is slightly above that of the room in which the experiment is being conducted. The increase in pressure within the chamber which results is indicated by the manometer. The withdrawal from the respiration chamber of samples for analysis, on the other hand, causes a reduction in pressure the extent of which can again be ascertained from the manometer.

Where the carbon dioxide of respiration is allowed to accumulate, as in a closed system, the increasing carbon dioxide concentration (partial pressure) in the chamber atmosphere and in the internal atmosphere of the fruit tends to drive this gas into solution in the tissues. Indications of this are afforded by the manometer, the record of thermometer readings indicating whether or not temperature changes have been operative in producing the results observed.

When a fruit, which has been held in a high concentration of carbon dioxide and low concentration of oxygen, is returned to normal air a rapid readjustment in the internal concentrations of these gases takes place. The apparatus described above can also be used to observe the nature and extent of these changes.

VII. OBSERVATIONS ON TEMPERATURE CHANGES

That fruits show marked changes in the amount of heat which they liberate at different stages during ripening is well known, and calorimetric methods have been devised by others by which these important changes can be followed with some degree of precision. In the present investigations the equipment necessary for such studies was not available nor was thermo-electric apparatus for the measurement of temperature. The temperature changes undergone by banana fruits when ripened at a steady temperature, particularly at a high temperature, are, however, so pronounced, that useful observations can be made using mercury-in-glass thermometers calibrated in 0.2° F.

The arrangement of apparatus is as follows. A respiration chamber of the type already described is used, the bung being bored so as to accommodate a lead-in tube and two thermometers. One of the thermometers is inserted longitudinally into the banana fruit contained in the chamber, and the other measures the air temperature in the respiration chamber. During experiments in which comparable respiration chambers and fruits are supplied with air at a constant rate, but of different controlled relative humidities, useful information can be obtained by comparing the records of air temperature in the storage room and respiration chamber and the temperature of the fruit flesh during ripening. The records shown in Fig. 2 may be cited in illustration. Discussion of this important aspect of respiration is postponed.

VIII. SUMMARY

The view is advanced that respiration studies should be conducted on as comprehensive a basis as possible. Measurements of the internal concentration of carbon dioxide and of oxygen and also of the amounts of carbon dioxide contained in the tissues, are considered essential adjuncts to the measurement of the rate of carbon dioxide liberated at the surface of the fruit, especially where changes in this rate are taking place.

Methods and apparatus are described, with special reference to the banana, for the measurement of (i) respiration rate, (ii) internal gas concentrations, (iii) pneumatic pressure within fruits and (iv) tissue content of carbon dioxide. Methods whereby observations may be made on gradients of gaseous concentration and on temperature changes during ripening are also described.

In each instance, the advantages and limitations of the methods described are discussed, and their applicability to other fruits indicated.

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Cytogenetical Studies in Oryzeae and Phalarideae

III. Cytological Studies in Phalarideae¹

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With Plate I and seventy-one Figures and two Diagrams in the Text

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INTRODUCTION

IT is now recognized that the cytological investigations of plants belonging to various species and genera form a useful adjunct to the morphological classification of the systematist. While cytology is concerned with the study of the germ plasm, taxonomy is based on the study of the similarities and differences in the morphological features of the adult forms which result from the germ plasm. Both these methods of approach to the study of speciation should supplement each other.

According to the modern systematists, the Phalarideae are a small tribe with six genera, Phalaris, Anthoxanthum, Hierochloa, Ehrharta, Microlaena, and Tetrarrhena. This was Bentham's (1881) classification. Difficulties, however, regarding the position of the last three genera had been felt by the earlier systematists. Swartz (1802) placed Ehrharta near Oryza. Nees (1841) included Ehrharta in the tribe Oryzeae along with Leersia, Maltebrunia, and Oryza. Kunth (1829, 1833) included the three genera Ehrharta, Microlaena, and Tetrarrhena in Oryzeae, while Trinius (1839) and Nees (1836) included them in the tribe Phalarideae.

From a morphological study of the spikelet these six genera could be classified into two groups, one containing the last three genera, and the other the first-mentioned genera. In the first group the first two glumes are smaller

¹ Part III of thesis for the Ph.D. degree of the University of London.

than the sterile lemmas, while in the second group they enclose the remainder of the spikelet. In *Oryza*, the sterile lemmas had been mistaken for glumes; Stapf (1898) recognized the two protuberances below the sterile lemmas as the glumes, and Arber (1934) from an histological study confirmed Stapf's observations. These observations bring the first group, *Ehrharta*, *Microlaena*, and *Tetrarrhena*, nearer to *Oryzeae* than to the second group in *Phalarideae*.

From a karyological point of view Avdulov (1931), by examining the somatic chromosomes of *Ehrharta panicea*, found that they were small and in multiples of twelve ($2n = 24$), and hence suggested the resemblance of the first group to *Oryzeae*. In view of the affinities indicated in the tribe *Phalarideae* to *Oryzeae*, a cytological survey of the tribe *Phalarideae* was undertaken and the results of the preliminary studies are reported in the present paper. The investigations are being continued with the availability of materials. The results include a study of thirteen species representing four genera, of which the chromosome numbers of seven species are recorded for the first time.

NATURE OF THE CYTOLOGICAL SURVEY

The chromosome studies in the four genera *Anthoxanthum*, *Phalaris*, *Ehrharta*, and *Microlaena* include the study of somatic mitosis, and in the first three meiosis for some species has been followed. The study of mitosis in general indicates the nature of the evolution of the chromosome complement in species and genera. The present investigations confirm Avdulov's observations regarding the resemblance of *Ehrharta* and *Microlaena* to *Oryzeae* and the complete lack of similarity between the first and second groups in *Phalarideae*: this is in conformity with the classification of the earlier systematists, and further studies in most of the species embracing the three genera should settle this definitely.

Changes of the following nature are inferred in the different species: (1) chromosome size, due to differences in the degree of contraction at mitotic metaphase, as in *Crepis* (Navashin, 1931); (2) numerical variation (polyploidy and aneuploidy); (3) structural changes, inferred from the morphology of the chromosome complement. The position of the spindle-fibre attachment region, which determines the arms of the chromosomes, the secondary constrictions and the satellites or trabants, indicates the chromosome type in a species, and any changes observed in the positions of these lead to the inference of translocation, fusion, or fragmentation according to the changes involved. (4) Another distinguishing feature of mitosis is the number of nucleoli formed in telophase. This is an evidence of the number of satellite chromosomes present in the complement (Heitz, 1931), and variation in number generally indicates polyploidy, secondary polyploidy, or aneuploidy.

More important is the study of meiosis, where changes not detectable in mitosis reveal themselves owing to the special condition of pairing and crossing-

over. Such changes are translocations, deletions, inversions, duplications, &c. Sometimes failure of pairing results not only from segmental non-homology but also is determined by gene mutation, as in *Zea Mays* (Beadle, 1930) and in rice (Ramanujam and Parthasarathy, 1935). Multivalent formation indicates the nature of polyploidy (autotetraploidy) and under particular conditions segmental interchange, as in *Oenothera*, *Tradescantia*, *Aucuba*, &c. Absence of multivalents in species with double the diploid complement favours allotetraploidy, the origin of which is hybridization of species, with different chromosome complements followed by doubling of chromosomes, as in *Primula Kewensis*, *Galeopsis Tetrahit*, *Spartina Townsendii*, *Iris versicolor*, &c.

Secondary association of chromosomes, a characteristic phenomenon of metaphase I and II of meiosis, especially in plants with small chromosomes, is an indication of the ancestral homology of the attracted chromosome bivalents. The study and analysis of the associations contribute to the inference of allotetraploidy or secondarily balanced allotetraploidy from the probable basic number of the species. Species which had been considered diploids have now been established as having been derived from a primary basic number, as in *Brassica* (Catcheside, 1934; Alam, 1936), in Pomoideae (Moffet, 1931), and in rice (Nandi, 1936, and Sakai, 1935).

Observations in the present study are sufficient to show that genotypic, numerical, and structural changes have occurred during the evolution of the species.

MATERIALS

Seeds of the several species in the different genera were obtained from the following places by Professor R. R. Gates, to whom my thanks are due.

Phalaris spp.: Museum d'histoire Naturelle, Paris; Waite Agricultural Research Institute, Australia; Cambridge Botanical Gardens; Plant Breeding Station, Aberystwyth.

Ehrharta spp. and *Microlaena* spp.: Department of Agriculture, Pretoria; Botanical Gardens, Melbourne.

My thanks are also due to Mr. C. E. Hubbard, of the Royal Botanical Gardens, Kew, for supplying me with seeds of various *Phalaris* spp. obtained from different places. I am also indebted to him for the identification of plants raised from seeds received, and for help in the taxonomy of the tribe.

The plants were raised at the Courtauld Genetical Laboratory, Regent's Park, London, during the summer of 1937.

CYTOTOLOGICAL TECHNIQUE

Root-tips from adult plants were fixed in Benda's fluid, 2 BE and Navashin's fluid. While Benda's and 2 BE gave good fixation for *Phalaris* and *Anthoxanthum*, Navashin's and 2 BE were found suitable for *Ehrharta* and *Microlaena*. For the comparison of chromosome complements, drawings were made from preparations in the same fixative, as it was found that marked difference

in size was noticeable in the same materials fixed in two different fixations. (Cf. Text-figs. 3 and 4 fixed in Benda and 2 BE respectively.) Good metaphase plates were obtained from materials collected between 11 a.m. and 12 noon.

For the meiotic stages, materials were collected between 10 a.m. and 11 a.m. Spikelets were dipped in Carnoy's (Semmens, 1937) for a few seconds and rinsed in water, then fixed in Navashin's fluid and 2 BE or medium Flemming. Instead of osmic acid, uranic acid was used with good results. While Flemming and 2 BE were found to be good for particular stages, Navashin's was uniformly good for the species examined. Aceto-carmine smears were used to determine the correct stages before the fixation of the material.

SOMATIC MITOSIS

The following table gives the list of chromosome number found so far in the different species examined, together with the numbers determined by previous investigators:

TABLE I
Chromosome Numbers in Phalarideae

Genus and species.	n.	2n.	Reference.
<i>Anthoxanthum odoratum</i> .	8	—	Marchal (1920)
	10	—	Kattermann (1931a)
	—	20+6f	Hunter (1934)
	—	20	Avdulov (1931)
	10	20	Author
<i>Phalaris canariensis</i> .	6	—	Kattermann (1931b)
	6	—	Church (1929)
	14	28	Nakajima (1933)
	6	12	Author
<i>P. brachystachys</i> .	6	12	"
<i>P. paradoxa</i> .	.	—	Avdulov (1931)
	.	7	14
<i>P. caerulescens</i> .	.	7	14
<i>P. Lemmoni</i> .	.	—	"
<i>P. tuberosa</i> .	.	14	Jenkin and Sethi (1932)
	.	—	Author
<i>P. arundinacea</i> .	.	14	Jenkin and Sethi (1932)
	.	—	Hunter (1934)
	—	28	Avdulov (1931)
	14	—	Church (1929)
	7	—	"
	—	28	Author
<i>P. minor</i> .	.	—	Avdulov (1931)
	14	28	Author
<i>Ehrharta erecta</i> .	.	12	24
<i>E. calycina</i> .	.	24	48
<i>E. longiflora</i> .	.	—	48
<i>Microlaena stipoides</i> .	.	—	48
			"

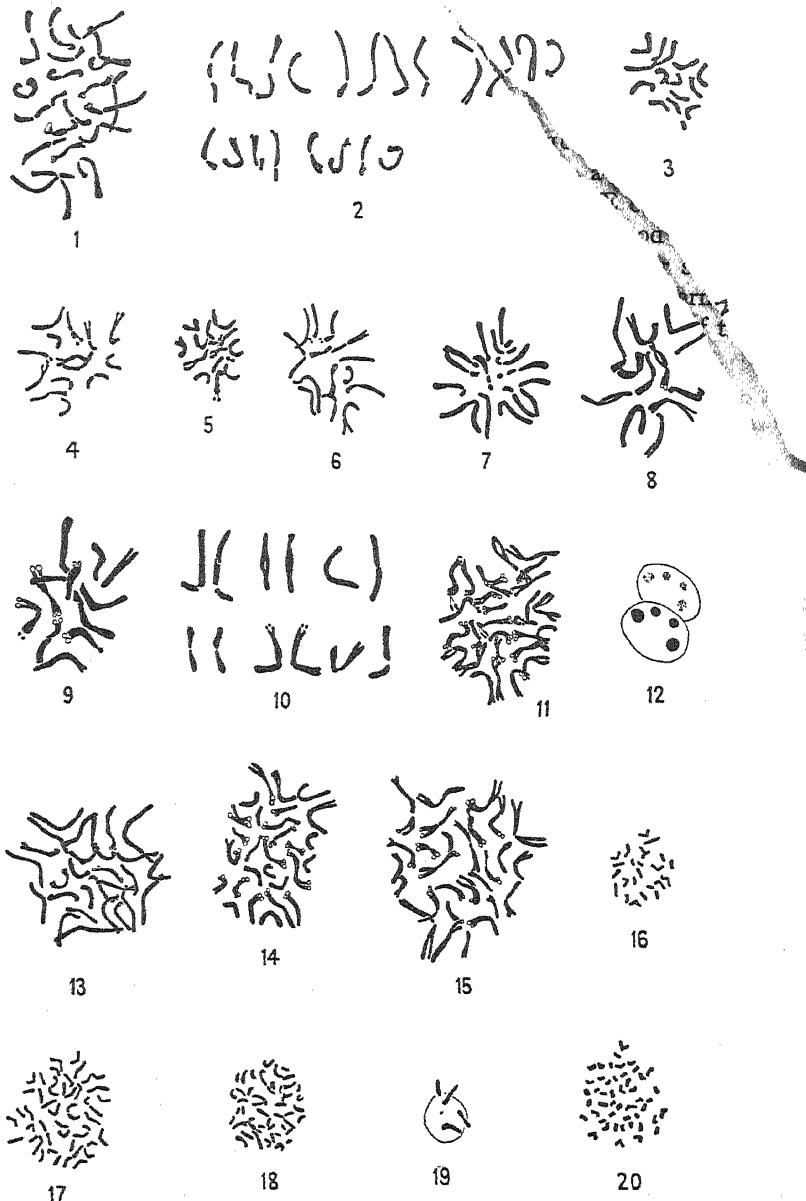
Anthoxanthum. Excepting one species, *Anthoxanthum odoratum* L., which is widespread throughout Europe and Asia, the others are limited mostly to

particular regions, as the Mediterranean, tropical Africa, Philippines, and S. Africa. *Anthoxanthum odoratum* L. ($2n = 20$).

Root-tips were collected from plants growing at Kew and also from plants raised from seeds received from Cambridge. The somatic chromosome number is twenty. Two pairs of large fragments and one pair of small fragments noted by Hunter (1934) were not observed in the present material. He considered these fragments to be similar to the extra chromosomal bodies in *Zea Mays* (Avdulov, 1933) and in *Paspalum stoloniferum* (Avdulov and Titova, 1933). It was difficult to get flat plates without foreshortening of chromosomes. Text-fig. 1 represents a fairly good plate and in Text-fig. 2 the chromosomes are roughly grouped according to size and morphology. It is possible to identify five types, each type being repeated four times. One type with secondary constriction is definite. There are two long types with sub-median constrictions and two small types. The homology is not very perfect, due not only to the chromosomes not being flat in one plane, but also probably to the fact that segmental interchange between non-homologous chromosomes has taken place, as could be seen by the behaviour of the chromosomes during meiosis. Meurman (1929) in *Aucuba*, which is also an autotetraploid in which this structural change has taken place, was able to identify eight types repeated four times. Probably the interchange had taken place between only small terminal segments without radically altering the morphology of the complement.

No satellites were observed and neither have they been reported in this species. Secondarily constricted chromosome types are not recognizable in the figures drawn by Hunter (1934) or Avdulov (1931). A secondarily constricted chromosome was first recognized as a triarticulate body by Delaunay (Lewitsky, 1931a), who claimed that the number of segments was characteristic of definite species. Nucleoli have been found to be organized at the secondary constrictions in quite a similar manner as in the satellited constrictions (Heitz, 1931). But Sato (1936) claims that the secondary constrictions have no connexion with the nucleolus and are essentially different from the satellited constrictions. Perhaps in the absence of satellited chromosomes, the secondarily constricted chromosomes behave as nucleolar chromosomes. From a morphological point of view, a secondarily constricted chromosome and a SAT-chromosome are similar except for the length of the segment distal to the constriction. In one case there is a definite chromosome segment, while in the other only a very small portion of it. It is quite probable that the SAT-chromosome represents a higher evolutionary type. Chen (1936) finds in Protozoa that the nucleoli develop each on a separate chromosome and are always non-terminal in origin. They generally surround a portion of the chromosome which is visible through the more transparent nucleolus: probably the constriction has evolved at this region of the chromosomes later from this primitive condition.

Phalaris. This genus is widely distributed. *Phalaris arundinacea*, known as the 'reed canary grass', is a hygrophilous species, widespread throughout the



All drawings were made at bench level with the aid of a camera lucida. An achromatic objective N.A. 1.3 was used in conjunction with Zeiss eye-piece K. 25 giving approximate magnification of 4,000 diameters. These were reduced to half size in reproduction.

TEXT-FIGS. 1-20. Fig. 1. *Anthoxanthum odoratum*. Somatic metaphase $2n = 20$. Fig. 2. *Anthoxanthum odoratum*. Chromosome morphology. Fig. 3. *Phalaris caerulescens*. Somatic

Northern Hemisphere as well as in isolated regions in NE. Tropical Africa and coastal regions of S. Africa. *Phalaris minor*, *P. brachystachys*, *P. tuberosa*, *P. paradoxa*, and *P. caeruleascens* are confined to the Mediterranean region, Arabia, N. and NE. Africa, while *P. canariensis* is native of the Western Mediterranean. *Phalaris angusta*, *P. Lemmoni*, *P. californica*, and *P. caroliniana* are native of temperate regions of N. America, especially in the west, with the exception of *P. angusta*, which has a wider distribution extending even to southern regions of S. America.

Phalaris caeruleascens Desv. ($2n = 14$) is often mistaken for *P. tuberosa*, as it resembles the same in most of its external morphological characters. An easy means of distinguishing them is by examining the outer glumes of the spikelet, the margin of which is entire in the latter, while in the former it is serrated. The chromosomes have median or nearly median constrictions. There is a pair of SAT-chromosomes whose satellites, usually small and spherical, are definitely larger and resemble a small segment of a chromosome. Text-fig. 4 represents the complement drawn from a preparation of the same material in Benda's, while Text-fig. 3 is from La Cour 2 BE. The chromosomes are definitely longer and more slender in Benda's fixation and the satellites are small, rounded, and double. This is a case where different conditions of fixation induce a change in chromosome contraction or spiralization. Cold treatment before fixation also has been found to induce such differences, as in *Crepis* (Delaunay, 1930).

Different sources of variation in size and shape of chromosomes are identified in fixed preparations. Navashin (1934), in *Crepis*, has recorded variations in size of chromosomes in embryonic and adult roots and in different regions of the same root. Such variations may be due to the differential effect of environment in different cells. Two cells in the same section (Text-fig. 5) showed chromosomes shorter and a little thicker than the normal type, which is also drawn from the same section in the same region. As the conditions of fixation are identical, the change in the chromosome size may be attributable to a cell mutation. The characteristic differences between species and between varieties of the same species are assumed to be due to genotypically controlled differences. Two clones of *Fritillaria ruthenica* (Darlington, 1936) were found to differ in the size of the chromosomes, the smaller type having smaller

metaphase $2n = 14$. 2 BE fixation. Fig. 4. *Phalaris caeruleascens*. $2n = 14$ Benda fixation. Fig. 5. *P. caeruleascens*. Chromosomes in a mutant cell. Fig. 6. *P. paradoxa*. Somatic metaphase $2n = 14$. Fig. 7. *P. Lemmoni*. Somatic metaphase $2n = 14$. Fig. 8. *P. canariensis*. Somatic metaphase. $2n = 12$. Fig. 9. *P. brachystachys*. Somatic metaphase. $2n = 12$. Fig. 10. *P. canariensis*. Chromosome morphology. Fig. 11. *P. arundinacea*. Somatic metaphase. $2n = 28$. Fig. 12. *P. arundinacea*. Somatic telophase showing four nucleoli. Fig. 13. *P. tuberosa*. Somatic metaphase. $2n = 28$. Fig. 14. *P. minor*. Somatic metaphase. $2n = 28$. 2 BE fixation. Fig. 15. *P. minor*. Somatic metaphase. $2n = 28$. Benda fixation. Fig. 16. *Ehrhartia erecta*. Somatic metaphase. $2n = 24$. Fig. 17. *E. calycina*. Somatic metaphase. $2n = 48$. Fig. 18. *E. longiflora*. Somatic metaphase. $2n = 48$. Fig. 19. *E. calycina*. Somatic prophase showing four chromosomes on the nucleolus. A satellite of one of the chromosomes is also seen on the nucleolus. Fig. 20. *Microlaena stipoides*. Somatic metaphase. $2n = 48$.

chromosomes than any other in *Fritillaria*. There are a number of cases known where change in the size of chromosomes of the entire complement are attributable to the action of the genotypes. Breslavetz (1929) in *Melandrium*, Thomas (1936) in *Lolium perenne*, and Ramanujam (1938) in *Oryza*. Lesley and Frost (1927) in *Matthiola* have shown that the length of the chromosomes at the first meiotic division is controlled by a single gene. It is quite probable that the marked change in the size of the chromosomes may have resulted from mutation in these cells, as cells in similar regions showed normal chromosomes. This is significant in connexion with the fact that different sizes of chromosomes in related species may be due to genetically controlled differences in different species. *Phalaris paradoxa*, an allied species, though showing more or less similar morphological features in the chromosomes, has definitely thinner and longer chromosomes which may be due to the altered genotype.

Phalaris paradoxa L. var. *praemorsa* Coss. and Dur. ($2n = 14$). The chromosomes are similar to those of *P. caerulescens* except for length and thickness. The chromosomes are drawn from preparations in 2 BE (Text-fig. 6). These almost resemble the chromosomes of *P. caerulescens* fixed in Benda (Text-fig. 4). The differences existing in species appear as though they can be stimulated by fixatives. One large pair with median constrictions, one satellited pair and one small pair, definitely smaller than the rest, are easily identifiable. Others have nearly median or submedian constrictions.

Phalaris Lemmoni Vasey ($2n = 14$) (Text-fig. 7). The chromosome complement is different from the two species of *Phalaris* described before. There are three pairs with subterminal constrictions, of which one pair is satellited, the satellites being in the shorter arm, one large pair with median constriction similar to that of *P. paradoxa*, and three pairs with definitely submedian constrictions. The changes involved in this complement are in the positions of the centromeres, which might have been brought about by fragmentation of one arm, resulting in the markedly unequal arms. The satellite attachment could change from a submedian to subterminal chromosome by translocation. This species appears to be an intermediate form in the evolution of twelve chromosome types found in *Phalaris canariensis* and *P. brachystachys*. This point will be discussed later. Another peculiar feature in the metaphase plate (Text-fig. 7) is the arrangement of chromosomes with the constrictions all lying in the periphery of the spindle, instead of being distributed evenly in the plate. This arrangement is characteristic of animal cells.

Phalaris canariensis L. and *P. brachystachys* Link ($2n = 12$). While the former species is a cultivated annual, the latter is wild. The chromosome complement in both is similar, except that in the latter the chromosomes are a little thicker and a little shorter (Text-figs. 8 and 9). Both have a pair with satellites and nearly median constriction, one pair with subterminal constrictions and the rest with nearly median or submedian constrictions. One pair with submedian constrictions is definitely longer than the rest (Text-fig. 10); this pair and the one with subterminal constrictions are easily recognized at

meiotic metaphase by their rod configurations in most of the cells studied. The chromosomes are definitely larger in size than the 14-chromosome types, which may be due to genotypic differences. It is probable that this 12 type is derived from the 14 type in *Phalaris Lemmoni* by the fusion of two pairs of chromosomes with subterminal constrictions to give one pair with median constrictions. Such fusion has been inferred in *Drosophila* (Gates, 1924), and later such fusions between the X-chromosome and the fourth chromosome, to give a J-shaped chromosome, has been obtained in *Drosophila* in translocation experiments (Painter and Stone, 1935).

Phalaris arundinacea L. ($2n = 28$). The material examined shows twenty-eight chromosomes (Text-fig. 11), while Church (1929) has reported fourteen and twenty-eight chromosomes ($n = 7$ and 14). Evidently this is a case of polyploidy within the species. From Jenkin and Sethi's (1932) observations regarding meiosis, the regular formation of bivalents without multivalents, as in the other polyploid races within the species, found in nature, is rather remarkable. It is quite probable that this form arose as an autopolyplid with free pairing of homologues and consequent partial sterility, but later had gradually evolved into an allopolyploid condition with regular bivalent formation and restoration of fertility. This should have been brought about by differentiation in the chromosomes of the duplicated set, by gene mutations or structural changes or both. Two pairs of satellite chromosomes could be distinguished in the complement and four nucleoli are formed in the somatic telophase (Text-fig. 12).

Phalaris tuberosa L. The chromosome number $2n = 28$ reported by Jenkin and Sethi (1932) is confirmed (Text-fig. 13). Four nucleoli are found at somatic telophase, though satellites or secondary constrictions could not be identified in the preparation.

Phalaris minor Retz. ($2n = 28$). An annual weed which is shorter in stature than either *P. tuberosa* or *P. caerulescens*. It has only one pair of SAT-chromosomes and the number of nucleoli at somatic telophase is also only two. This species is presumably an allotetraploid, as it forms fourteen bivalents at meiotic metaphase except for an occasional quadrivalent. The absence of another SAT-pair is perhaps due to loss of a satellite by mutation and the evolution of a single satellite pair by segregation, as in rice (Ramanujam, 1937). The chromosomes drawn from the preparations in La Cour 2 BE and Benda (Text-figs. 14 and 15) show the effect of different fixatives on chromosome size. The satellites are not visible in materials fixed in 2 BE.

Ehrharta. This genus is distributed in S. and E. Africa and one species, *E. abyssinica*, is found also in Nilgiri, S. India, and in Madagascar.

Ehrharta abyssinica Hochst. has thus a wider distribution than *E. erecta* Lam., *E. longiflora* Sm., and *E. calycina* Sm., which are confined to S. Africa.

The chromosomes of this genus from the three species examined are in marked contrast to those of *Phalaris* and *Anthoxanthum* (Text-figs. 16, 17, 18).

These compare with Oryzeae, although they are slightly larger, and they are also in multiples of twelve as in *Oryza*. No satellites could be made out. In *Ehrharta calycina* four chromosomes were found to be on the nucleolus in somatic prophase (Text-fig. 19). Though the chromosomes of *Oryza* are in multiples of twelve, it is now established that this is a secondarily balanced basic number for a new line of evolution from the primary basic number 5. There is also good evidence of secondary association in the diploid species, *Ehrharta erecta* ($2n = 24$), indicating that the haploid set of twelve in this genus is also a secondarily derived number, but sufficient plates were not available to establish the maximum association.

Microlaena. The distribution of this genus is confined to Australia, New Guinea, Tasmania, and New Zealand. The chromosome number $2n = 48$ is determined for the first time in this genus for the species *M. stipoides* R. Br. (Text-fig. 20). This is known as 'weeping grass'. The chromosomes are smaller than in *Ehrharta* but appear thicker, which is due to Navashin's fixation. The fixation was not good in other fixatives tried.

MEIOSIS

The following available materials were examined for the study of meiosis: (1) *Anthoxanthum odoratum* L., (2) *Phalaris canariensis* L., (3) *P. caerulescens* L., (4) *P. paradoxa* L., (5) *P. brachystachys* Link, (6) *P. Minor* Retz, (7) *Ehrharta erecta* Lam., (8) *E. calycina* Sm. In all the species examined early prophase stages could not be studied, but the behaviour of chromosomes from diakinesis onwards is described.

1. *Anthoxanthum odoratum* ($2n = 20$). Kattermann (1931) has described the reduction divisions in this species and has found that instead of forming ten bivalents, a number of different chromosome associations in rings and chains occur. This is perhaps the reason that Marchal (1920) counted only eight bivalents in the meiotic metaphase. The associations of these polyploid chromosomes are terminal. The maximum number of chromosomes in one catenation was twelve, and out of fifty cells examined, only one showed all the ten as bivalents. He infers that autopolyplodity combined with segmental interchange should account for the multivalent formation.

From a study of mitosis it was possible to identify four sets of chromosomes, indicating that the species is a tetraploid. On that basis not more than four chromosomes should associate during meiosis. But associations of more than four were common. In diakinesis the chromosomes were found to be attached end to end, which was easily discernible in some cases as the regions of attachment were thin and drawn out (Text-fig. 21), while in other cases the ends were fused so that it was difficult to make out the number in each catenation (Text-fig. 22). The chromosomes were spread out in the drawings to make the individual associations clear. The associations of chromosomes were found to be similar to those described by Kattermann (1931). The following associations were observed in the cells at diakinesis and metaphase:

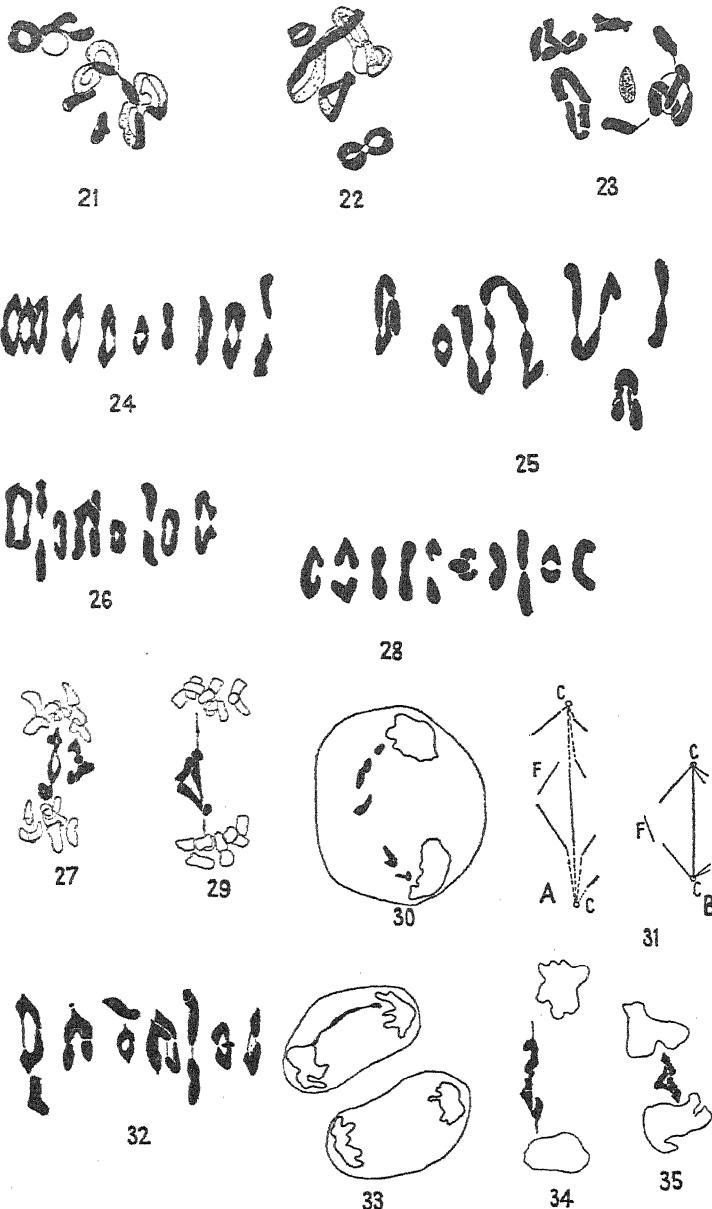
TABLE II
Chromosome Catenations in Anthoxanthum odoratum

Catenations.	No. of cells.
(12) - 4(2)	1
(10) + 2(4) + (2)	1
(10) + (4) - 3(2)	1
2(6) - 4(2)	1
(6) - 2(4) - 3(2)	1
(6) + (4) + 2(3) - 2(2)	1
2(4) - 6(2)	3
(4) - 8(2)	3
10(2)	1
	13

In metaphase the multiple associations in most of the cases were orientated in zigzag fashion, giving regular disjunction, showing that the mechanism bringing about this orientation is similar to that of *Oenothera* (Text-fig. 24). Occasionally adjacent chromosomes pass to the same pole, which will give rise to deficiency or duplication if the chromosomes were interchanged chromosomes (Text-fig. 25). A cell shows non-orientation of a chain (Text-fig. 26) which will evidently result in numerical non-disjunction, which is not rare in interchange heterozygotes.

At diakinesis there was found to be an association between the chromosomes and the nucleolus. In one case, the free ends of a bivalent were found to be attached to the nucleolus. Text-fig. 23 shows two chromosomes connected by a thin thread, one of which is on the nucleolus and in the same nucleus, one end of a chain of four is also on the nucleolus. In Text-fig. 21 it is found that a ring of six is attached by the end of one of the chromosomes to a chain of six, giving an association of twelve, the maximum number of chromosomes in single chain. In another case the end of a bivalent is connected to the ends of a second bivalent (Text-fig. 22), giving the appearance of a quadruple chiasma, which is often hard to distinguish from distal interlocking (Catchside, 1932). Occasionally lagging bivalents are noticed (Text-fig. 27) with interstitial chiasmata.

End-to-end association of chromosomes giving rise to configurations of more than four chromosomes expected on the basis of autotetraploidy could only take place on the basis of terminal homology in otherwise non-homologous chromosomes. This is possible, according to Belling (1925), by segmental interchange between non-homologous chromosomes. The origin and significance of segmental interchange in the evolution of species is discussed elsewhere. One peculiarity connected with this species is that it is a tetraploid like *Aucuba*. Whether segmental interchange has taken place in the diploid or after duplication in the tetraploid cannot be determined without examining the diploid species of this genus. The only diploid species so far examined is *Anthoxanthum aristatum*, $2n = 10$ (Avdulov, 1931), but



TEXT-FIGS. 21-35. Figs. 21-6, 28, and 32, drawings are spaced to show the associations. Fig. 21. *Anthoxanthum odoratum*, diakinesis, a chain of 6 connected to a ring of 6. Fig. 22. *A. odoratum*, diakinesis showing the association of $(8)+2(4)+2(2)$. Fig. 23. *A. odoratum*, diakinesis showing the association of $(6)+2(4)+3(2)$. Fig. 24. *A. odoratum*, side view of metaphase I, a ring of 6 and 7 bivalents. Fig. 25. *A. odoratum*, side view of metaphase I,

unfortunately its meiosis was not studied. This material could not be obtained for the present study. If the diploid ancestor of *Anthoxanthum odoratum* had all the chromosomes connected in one ring at the reduction division, this could be represented in the following way, assuming each chromosome had the end segments different.

A	B	C	D	E	F	G	H	I	J
B	C	D	E	F	G	H	I	J	A

The chromosome complement of the tetraploid plant derived from the duplication of the above chromosomes should be

AB,BC,CD,DE,EF,FG,GH,HI,IJ,JA
AB,BC,CD,DE,EF,FG,GH,HI,IJ,JA.

It is clear that ten bivalents can be formed as each chromosome has its mate, and as there are four similar ends of attraction for each segment of a chromosome there are possibilities of rings or chains of different catenations with a maximum of twenty chromosomes in a ring or a chain being formed depending upon chance. From the analysis of the configurations of groups examined by Kattermann and by myself, not more than twelve chromosomes were involved in a single association, showing thereby that segmental interchange had not taken place between all the chromosomes in the diploid. Probably only six chromosomes were united in a ring in the diploid, owing to two interchanges having taken place and one of the chromosomes being involved in both the interchanges. The duplication of the chromosomes in such a diploid could then give the ring or chain of twelve chromosomes. Text-fig. 28 represents a rare instance of ten bivalents.

Another interesting feature in this species, not recorded previously, is the presence of inverted segments in a single pair of chromosomes. This structural change could be detected either in the prophase pairing (McClintock, 1931) or in anaphase I and II by the presence of a chromatid bridge and fragment. Although it was not possible here to observe the inverted pairing of the inverted segments at pachytene, the presence of dicentric bridges with acentric fragments at anaphase may be taken as an evidence that such pairing had taken place followed by crossing-over in the inverted segments. The rare occurrence of the bridge and fragment in the material indicates either (1)

a chain of six not orientated. Association $(6)+(4)+2(3)+2(2)$. Fig. 26. *A. odoratum*, side view of metaphase I, a chain of 4 and a ring of 4 plus 6 bivalents. Fig. 27. *A. odoratum*. Anaphase I showing 2 lagging bivalents. Fig. 28. *A. odoratum*, side view of metaphase I, 10 bivalents. Fig. 29. *A. odoratum*. Anaphase I. A bridge with thin thread connexions at either end and a fragment. Fig. 30. *A. odoratum*. Anaphase I. Bridge broken, with a fragment at equator. The sister chromatids are separated due to tension. Fig. 31 A. *A. odoratum*. The bridge at anaphase I with sister chromatids separated due to the differential elongation of the connecting threads to the centromere. Fig. 31 B. *A. odoratum*. Diagrams (cf. Fig. 53) showing the normal bridge at anaphase I. Fig. 32. *A. odoratum*, side view of metaphase I showing the centromere and unpaired chromosomes. Association $2(4)+5(2)+2(1)$. Fig. 33. *A. odoratum*, anaphase II, chromatin bridge in one daughter-cell. Figs. 34 and 35. *A. odoratum*, anaphase I, bivalent configurations, giving unequal chromatids.

that the relatively inverted segment involves only a small region of the chromosomes and is due to the rare crossing-over, the occurrence of which alone in the inverted region gives rise to the bridge at anaphase I; or (2) that non-homologous association (McClintock, 1933) takes place in the inverted region with the absence of crossing-over. For if the inversion lies between two homologous pairing segments, it is necessary that either (1) the inverted segments do not pair, or (2) they pair, giving rise to non-homologous association. It is not possible to identify any of these in the prophase.

The consequences of crossing-over in the relatively inverted segments have been analysed by Richardson (1936), and the types relating to present observations in this species and in *Phalaris brachystachys* are represented later in the paper. The peculiarity of the bridge (Text-fig. 29) is that the ends are drawn out as thin threads reaching the chromosome groups near the poles. Text-fig. 30 shows that the bridge has broken with the fragment left at the equator and the sister chromatids are also separated, showing the effect of tension. Evidently this should be the cause of the thin connecting thread in Text-fig. 29. The diagram indicates the probable configuration of this bridge bivalent and a normal bridge bivalent (Text-fig. 31 A and B). The unusual tension in the regions on either side of the equator indicates the nature of the forces governing the anaphase separation. At early anaphase in normal bivalents it is observed that the centromere leads, as the name (*Leitkörperchen*) given to it by its discoverer (Metzner, 1894) indicates. In Text-fig. 32 also (Pl. I, Fig. 1) it is seen as a very small body ahead of the chromosomes, directed towards the pole. The dicentric chromatid, being connected between the two repelling centromeres, gives an indication of the nature of the forces governing the separation. If the repelling forces were uniform between the centromeres, throughout the anaphase separation, the chromatids connecting the centromeres will thin out or uncoil uniformly. But it is seen in this case, as well as in the case of *Phalaris brachystachys* (Text-fig. 59), that this is uneven and the thinning is on either side of the equator. This suggests another force special to this region which is perhaps due to the elongation or axial stretching of the spindle in this region, as put forward by Belar (1929), Darlington (1936), and Alam (1936). Upcott (1937) was the first to confirm this by observation on the dicentric chromatids in *Tulipa*. In the particular case referred to here the stretching affects the free chromatids as well. That free sister arms can separate at the point (centromere) where it is assumed they are each attached to the centromere is rather extraordinary. It is probably due to the differential elongation of the threads connecting the sister chromatids with the centromere.

The bridges formed evidently break before the formation of the cell-wall (Text-fig. 29). The probability in the case of Text-fig. 27 is that a restitution nucleus may be formed. Text-fig. 33 shows a chromatin bridge in the anaphase II.

The occurrence of configurations (Text-figs. 34 and 35) in anaphase is another feature which suggests that unequal chromatids are formed. These

could arise in three ways: (1) If the chromosomes comprising the bivalent were themselves unequal (Diagram I A), (2) if the centromere is included in an inverted segment (Diagram I C), and (3) if the homologous segments were relatively dislocated in the bivalent (Diagram I B).

Crossing over in all these three cases will give rise to unequal chromatids.

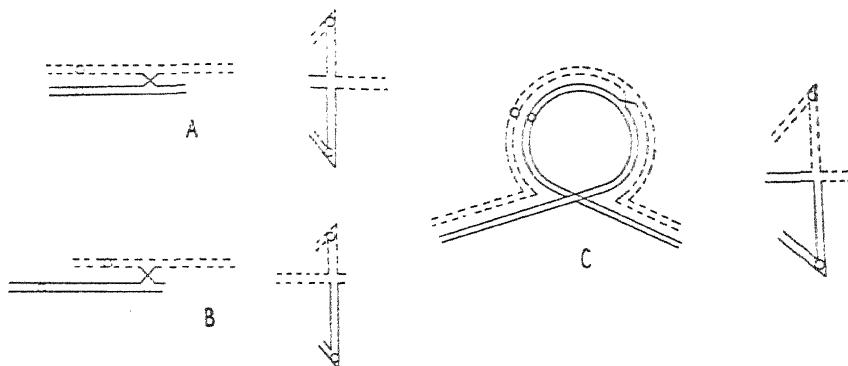


DIAGRAM I. Showing the origin of unequal chromatids from (A) unequal bivalent, (B) relatively dislocated segments, and (C) centromere being included in the inverted segment (cf. Figs. 34, 35, 60).

In *Anthoxanthum* it is not possible to identify an unequal bivalent such as Kattermann (1931) mentions and figures in metaphase I. The fact that this species is an interchange heterozygote is an evidence in favour of such bivalents existing, and also for relatively dislocated or inverted segments being present. It is not possible to determine the exact nature of the change involved, though any of these would account for the phenomenon observed. The occurrence of unequal chromatids is also noticed in *Phalaris brachystachys*, where, from the shape and size of the bivalents, there is no evidence in favour of unequal bivalents being present. The importance of reciprocal translocations in the evolution of species and the special mechanism favouring the permanence of such changes in nature has been dealt with elsewhere.

Phalaris caerulescens L. ($2n = 14$). In pachytene in favourable preparations it was possible to find a pair of threads connected subterminally with the nucleolus (Text-fig. 36). The function of satellite chromosomes in organizing the nucleolus has been clearly established by Heitz (1931). The exact morphological connexion between the nucleolus and the chromosome was first pointed out by Latter (1926). McClintock (1934) inferred the existence of a nucleolar organizer staining deeply to acetocarmine, lying at the end of the chromosome proper, where the nucleolus and the constriction are developed. Gates (1937) has given a succinct review of literature regarding the relationship of the nucleolus and chromosomes. McClintock's work showed that, as a result of X-raying, the organizer in one cell was divided into two parts, one of which was transferred to another chromosome by interchange. These parts were



TEXT-FIGS. 36-52. Fig. 36. *Phalaris caerulescens*. Pachytene showing the pair of threads attached to the nucleolus subterminally. Fig. 37. *P. caerulescens*. Diakinesis showing the attachment of the bivalents to the nucleolus. Fig. 38 A. *P. caerulescens*. Side view of metaphase I, bivalents spaced in the drawings. 5 rings and 2 rods. Fig. 38 B. *P. caerulescens*. Side view of metaphase I, bivalents spaced in the drawings, all rings. Fig. 38 C. *P. caerulescens*

found to have different activity. The smaller distal part containing the satellite which was transferred gave rise to the larger nucleolus, the larger proximal part left in the original chromosome gave a smaller nucleolus. The fact that the smaller part of the nucleolar organizing body and the satellite together gave rise to a larger nucleolus leads to the inference that the satellite may also be concerned in the organization of the nucleolus. But in the spores deficient in satellite and containing only a segment of the nucleolar organizer, a large nucleolus developed, showing that in the absence of the satellite the nucleolar organizing body can form a normal sized nucleolus. Navashin (1927, 1934) observed in *Crepis* the phenomenon of amphiplasty or an apparent inability of one of the parental satellited chromosomes to function in the hybrid. He found that in certain *Crepis* crosses a satellite normally present in one of the contributing complements did not appear in the hybrid, and that one instead of two normal nucleoli were formed in the mitotic telophase. This evidently indicates the importance of the satellite or the connecting thread or both in the organization of the nucleolus, or it has to be assumed that the nucleolar organizer is rendered inactive by hybridity. In rice (Ramanujam, 1937), loss of a satellite by mutation was inferred in the evolution of varieties with two nucleoli from the varieties with four. Warmke and Johansen (1935) have reported that *Trillium ovatum* was distinguished from an allied species, *T. chloropetalum*, by the presence of a satellite in the former and its absence in the latter.

At diakinesis the attachment of the bivalent to the nucleolus was still recognizable, the satellited portion being present as dark round bodies lying in a vacuole developed in the nucleolus (Text-fig. 37). The bivalents were mostly in the form of rings, but bivalents with a single chiasma or three chiasmata were not infrequent. Analysis of ten nucleoli showed that the chiasma frequency per bivalent was 2·21, and the terminalization coefficient 0·80. While at metaphase I, analysis of sixty cells showed that the terminalization was complete, with a chiasma frequency of 1·49 per bivalent. Only rings and rods

Side view of metaphase I, bivalents spaced in the drawings, 5 rods and 2 rings. Fig. 39. *P. caeruleascens*. Polar view of metaphase I, showing 7 bivalents. Fig. 40. *P. caeruleascens*. Diakinesis showing a chain of 4 chromosomes and 5 bivalents. Fig. 41. *P. caeruleascens*. Diakinesis showing side view of metaphase I, non-orientation of a ring bivalent. Fig. 42. *P. caeruleascens*. Diakinesis showing side view of metaphase I, non-orientation of a rod bivalent. Figs. 43-6. *P. brachystachys*. Stages from diakinesis to metaphase, showing the disposition of the bivalents during the determination of the poles. Fig. 47. *P. brachystachys*. Side view of metaphase I, non-orientation of one rod bivalent. Fig. 48. *P. canariensis*. Side view of metaphase I, bivalents spaced in the drawings. Fig. 49. *P. canariensis*. Unpaired threads near the attachment to the nucleolus at pachytene. Fig. 50. *P. canariensis*. Nucleolar chromosomes at diplotene (A and B) the chromosomes are drawn out into thin threads near the attachment. (C to F) show that the distance of the chromosome arms on the nucleolus depends upon chiasma formation. (See text.) Fig. 51. *P. brachystachys*. Diplotene, showing the loop in one of the bivalents, which are spaced in the drawing. Fig. 52 A. *P. brachystachys*. Side view of metaphase I, showing the exceptional bivalents at the right end. Fig. 52 B. *P. brachystachys*. Side view of metaphase I (in both the bivalents are spaced in the drawing). Fig. 52 C and D. Polar view of metaphase I.

with terminal attachments were noted. The theoretically possible range of variation in the proportion of rod to ring bivalents is 0/7 to 7/0. A count of sixty cells showed the following distribution of rod bivalents:

No. of cells	Rod bivalents							
	0	1	2	3	4	5	6	7
	2	3	10	11	14	17	3	0
Mean ± 3.58							0.19	

These data show that the chances of ring-bivalent and rod-bivalent formation are equal. This is quite in harmony with the morphology of the chromosomes, as all the seven pairs have median or nearly median constrictions, so that the slipping off of the chiasma on either of the arms is purely determined by chance. Text-fig. 38 represents side views of metaphase I bivalents from three cells. Text-fig. 39 shows the polar view of the seven bivalents with six in the periphery and one in the centre, which is the commonest arrangement observed and is in agreement with the stable form of floating magnets (Meyer, 1879).

In two cells in diakinesis, end to end association of four chromosomes giving a chain, was observed. This is an evidence of end homology in non-homologous chromosomes, involving small terminal segments; the rare occurrence of this configuration indicates that a small translocation has occurred in the pre-meiotic cell (cf. Darlington, 1933, in *Secale*).

Another feature observed during metaphase I was the delay in orientation of a bivalent in the equatorial plate. The regular anaphase behaviour of the bivalents without lagging and the absence of different numbers of chromosomes in metaphase II cells indicate that the unorientated bivalent later gets to the equator and divides regularly. This delay in orientation is considered by Darlington (1937) as due to increased distance between the centromeres with consequent decrease in repulsion, and he thinks that repulsion is the effective agent in orientation. A particularly long bivalent with submedian centromeres in *Phalaris brachystachys* and *P. canariensis* forms a chiasma at the distal end of the long arms in most of the cells at diakinesis (Text-fig. 43). Though the distance between the centromeres in such a bivalent should be greater than in the ring bivalents with consequent weaker repulsion, no such failure of orientation is noticed. In *P. minor* in two cells a particular long bivalent with median constriction and connected only by one end is orientated in the metaphase plate, though it is crowded with fourteen bivalents (Text-fig. 66).

In the cases of delay reported here, the bivalent concerned is either a ring or a rod (Text-figs. 41 and 42), and so these instances show that the delay in orientation must be due to different causes. Observations of the short stage between diakinesis and metaphase show that the movements of the bivalents towards the equatorial plate can be analysed as follows: (1) The angular or rotational movement orientating the bivalent with the axis of the spindle or

the centromeres with the axis of the poles. (2) Translatory movement to the equator, due perhaps to the repulsion of the poles and the centromeres. Both these movements will lead all the bivalents to crowd at the centre of the plate, but the mutual repulsion between the bivalents keeps them spread out on the plate. The delay in orientation is presumably caused by the disposition of the axis of the centromeres in the bivalents during the disappearance of the nuclear membrane and the determination of the poles. Those whose axes are in the direction or nearly in the direction of the poles move quickly to the equator, while those whose axes are perpendicular are delayed. (Text-figs. 44 to 47 show the stages between diakinesis and metaphase and indicate how such a disposition may cause a delay in orientation.)

Phalaris canariensis L. ($2n = 12$). The meiotic chromosome number ($n = 6$) recorded by Church (1929) and Kattermann (1931) is confirmed in the present observations. The nature of the bivalents (Text-fig. 48) is quite similar to that of *P. brachystachys* described next. The anaphase behaviour is quite regular, unlike *P. brachystachys*, where structural changes are inferred. At pachytene it was found that the nucleolar chromosomes were unpaired near the attachments to the nucleolus (Text-fig. 49 and Pl. I, Fig. 2). At early diakinesis the nucleolar chromosomes are still connected with the nucleolus. In some cells the connexion between the attached portion of the chromosome and the main body is stretched out and thin (Text-fig. 50, A and B). The attachments are close together or further apart on opposite edges of the nucleolus, depending evidently upon where chiasma formation has taken place. If the chiasma is nearer the attachment, they are close together (Text-fig. 50 C). If it is distal to the attachments, they are farther apart (Text-fig. 50, D and E), and in some cases one of the arms slips off the surface of the nucleolus (Text-fig. 50 F). Upcott (1936) finds in *Eremurus*, when one arm is attached, the other arm has the appearance of being repelled from its homologue, which she considers as probably due to the nucleolus having the same surface charge as the chromosomes and exerting a repulsion force. Although the free arm does not show any reflection from the other in the present case, the presence of some sort of surface charge is to be inferred, as could be seen by the stretching of the chromosome into a fine thread near the attachments. The presence of a surface charge similar to that of the chromosomes has been inferred by McClendon (1910), who found that on passing an electric current through the living roots, the nucleolus and chromosomes move towards the anode, indicating that they bear the same negative charge. That the chromosomes are negatively charged has been confirmed in the recent experiments of Churney and Klein (1937), who found on passing an electric current through the salivary gland cells of the dipteran *Sciara coprophila*, the nucleus as a whole moved towards the cathode (positive charge) whereas the chromosomes contained in it moved towards the anode (negative charge).

Another strange feature is that while the satellites in somatic chromosomes are so small and sometimes identified with difficulty, the size and proportions



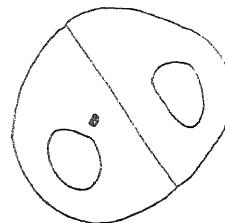
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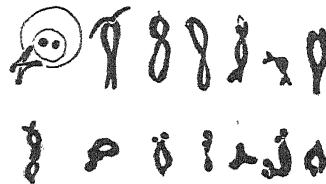
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67

TEXT-FIGS. 53-68. Figs. 53 and 54. Anaphase I. Dicentric chromatid bridge and fragment. Fig. 55. *Phalaris brachystachys*. Bridge broken and fragment at the equator. Fig. 56. *P. brachystachys*. Fragment left in cytoplasm. Fig. 57. *P. brachystachys*. Anaphase I. Dicentric loop chromatid—the fragment is near the upper pole. Fig. 58. *P. brachystachys*. Metaphase II. Polar view. The fragment is in one cell and a chromatid is seen split at the end. Fig. 59. *P. brachystachys*. Dicentric chromatid bridge with stretching of chromosomes.

of the attachments to the nucleolus at diakinesis are considerably larger when compared with the rest of the chromosome. It is concluded by McClintock that the nucleolar organizer is at the end of the chromosome proximal to the satellite, but it is possible that the stretched region may not be really the satellite stalk, but may represent a region proximal to the end of the chromosome, while the attached region may include the end of the chromosome, together with the satellite, the thread having been withdrawn.

Phalaris brachystachys Link ($2n = 12$). This wild species of *Phalaris* is particularly interesting in that it closely resembles *P. canariensis* both in external morphology as well as in chromosome morphology and number. Taxonomically the only difference consists in the sterile lemmas being shorter in *P. brachystachys*. Cytologically, the difference is clearly noticeable in anaphase I, and indicates the presence of inverted segments in the chromosomes which distinguishes this species from *P. canariensis*. Two plants examined both showed the existence of inversions.

In early diplotene, inversion pairing was observed in one cell by the characteristic loop (Text-fig. 51). Such loops were observed in pachytene in inversion heterozygotes by McClintock (1931) in *Zea*, Darlington (1930) in *Chorthippus* and *Stauroderus*, and Upcott (1937) in *Tulipa*. In the salivary gland chromosomes inversion pairing, giving rise to a loop, was noted by Koller (1936) in race hybrids of *D. pseudo-obscura*.

At metaphase, inversions are not usually recognizable or inferred, except when the individual chromatids remain distinct, as in *Paeonia* (Dark, 1936). The appearance of an exceptional bivalent (Text-fig. 52A) suggests that it may be the result of inversion pairing. As the individual chromatids could not be made out, the exact configuration is not clear. Text-fig. 52 A and B show side views of the configurations of metaphase in bivalents from two cells; and Text-fig. 52 C and D show the polar view from two cells.

At anaphase there is no doubt about the occurrence of crossing-over in the inversions. The cells showing the results of such crossing-over are rare, but the presence of an inversion in one of the chromosome pairs is inferred with certainty. Text-figs. 53 and 54, and Pl. I, Figs. 3 and 4, show the bridge and the fragment. In one case the fragment lies across the bridge in the centre, and in the other near one of the free arms. In late anaphase the bridges

near the attachment constrictions at either end. Fig. 60. *P. brachystachys*. Metaphase I bivalent configurations giving unequal chromatids—each drawn from a separate cell. Fig. 61. *P. brachystachys*. Abnormal mutant P.M.C. chromosomes are thin and long like somatic chromosomes. Fig. 62. *P. paradoxa*. Side view of metaphase I. 7 bivalents spaced in the drawings. Fig. 63. *P. minor*. Diplotene bivalents drawn separately. Note crossing-over between a pair of parental chromatids in the nucleolar chromosomes. Fig. 64. *P. minor*. Polar view of metaphase I. 14 bivalents. Fig. 65. *P. minor*. Side view of quadrivalents at metaphase I, each drawn from a separate cell. Fig. 66. *P. minor*. Side view of metaphase I, the bivalents are spaced in the drawing. In the long rod bivalent, the arms at either end are ahead of the constriction. Fig. 67. *P. minor*. Early anaphase, showing that 7 bivalents have separated before the others. Fig. 68. *P. minor*. Interlocking of a bivalent in an association of 4.

evidently get broken, as some cells show the fragment without the corresponding bridge (Text-fig. 55). In one cell a closed loop chromatid is formed and the acentric fragment is identified near the pole. This loop chromatid gives rise to a second division bridge. The acentric fragment is usually not included in the telophase nucleus (Text-fig. 56). When it does get included, as in Text-fig. 57, it is identified in the second division (Text-fig. 58). If the bridges are long, due to longer proximal segment to the inversion, they do not break, but persist in the second division, the cell wall after the first division passing right across the bridge (Sax, 1937, and Upcott, 1937). Bridges in the present material are definitely short, showing that the inversion region is proximal; and that it involves only a short segment is inferred from the rare occurrence of crossing-over giving rise to the bridge.

The significance of the occurrence of the bridge and the fragment was first inferred by McClintock (1933) in irradiated *Zea Mays* as cytological evidence for crossing-over in the inverted segment. Smith (1935) in *Trillium* found in addition to the bridge and the fragment in the first division a loop chromatid in the first anaphase which gave a bridge in the second anaphase. The first type he showed could arise from a single crossover in the inverted region, while the latter arose where there were at least two crossovers, one in the inverted region and the other in the region proximal to the inversion and in which one of the chromatids is involved in both the crossovers. A full analysis of crossing-over and chromatid relationship and the consequences in first anaphase in inversion hybrids has been dealt with by Richardson (1936) and Darlington (1936 and 1937). Only those which are relevant to the results obtained in the present study are discussed.

A dicentric chromatid bridge and an acentric fragment. This can arise in first anaphase by (1) a single crossover in the inversion (Diagram II A); (2) with two crossovers in the inversion in which one chromatid is involved in both the crossovers. In Diagram II B one crossover is between chromatids 2 and 4, and the second crossover is between 1 and 4; (3) one crossover in the inversion and one proximal to the inversion. The chromatid relationships between the crossovers is (a) that the same two chromatids are involved in both the crossovers (Diagram II C), or (b) that both the chromatids involved are different in the two crossovers (Diagram II D).

A loop chromatid and fragment in first anaphase. This can occur with two crossovers, one in the inversion and one in the region proximal to the inversion, in which only one chromatid is involved in both the crossovers (Diagram II E). This loop chromatid and consequently a second division bridge is a sure indication of a proximal homologous segment beyond the inverted region.

From the diagrams it may be noted that the size of the acentric fragment and the dicentric bridge will be constant, regardless of the position of crossing-over in the inverted region. The bridge is subjected to different degrees of tension at anaphase, so that its true length is difficult to estimate, but the size of the acentric fragments can be used as a fair measure for judging the size

of the inversion. Depending upon the position of inversion in the chromosome arm, fragments varying from small size to nearly double the length of the chromosome arm could be obtained. The length of the fragment increases

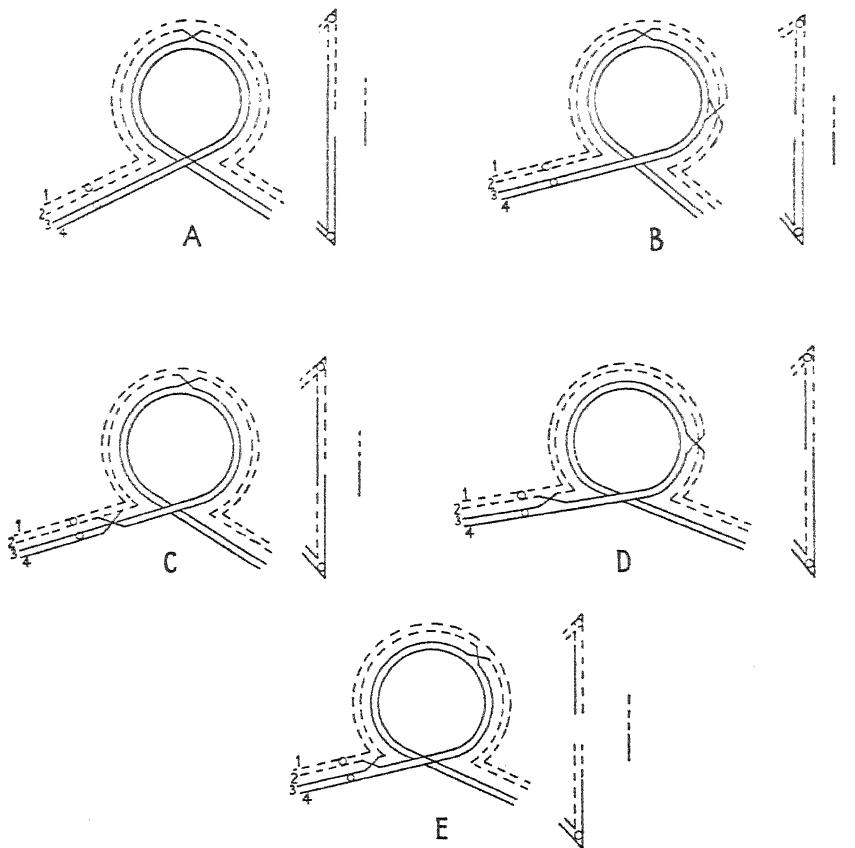


DIAGRAM II. Showing the origin of dicentric chromatid and fragment by crossing-over in inversion pairing. A. One crossover in the inverted region (cf. Figs. 53, 54). B. Two crossovers in the inverted region, one between (1 and 4) chromatids and the second between (2 and 4) (cf. Figs. 53, 54). C. One crossover in the inverted region and one in the segment proximal to the inversion. The same two chromatids are involved in the crossovers (cf. Figs. 53, 54). D. The chromatids involved are different in the two crossovers. E. A loop chromatid and fragment at anaphase. Two crossovers involved, one in the inversion and the other in the segment proximal to the inversion region. The same chromatid is involved in both the crossovers (cf. Figs. 33, 57).

with the proximity of the inversion to the centromere end. The uniform size of the fragments observed both in *Phalaris* and *Anthoxanthum* indicates the presence of a single inversion in a particular bivalent. The occurrence of different sizes of fragments would indicate the presence of more than one inversion. But Sax (1937) considers probable the occurrence of different sizes

of fragments from the same inversion, either by the occurrence of inverted crossovers in non-homologous straight association of inverted parts, or by crossing-over in loop pairing, which may shift throughout the length of the chromosome arm, extending to non-homologous regions as well. Whether a crossover can take place in non-homologous pairing parts is doubtful. At anaphase one of the bivalents is occasionally found to give rise to chromosomes in which one of the chromatid arms is longer than the sister arm. The configuration giving rise to this condition is shown in Text-fig. 60. The segmental constitution which would give rise to unequal chromatids has already been discussed.

Observations during diakinesis show that a particular long bivalent with submedian constriction and another shorter bivalent with subterminal constriction, in most of the cells, form only one chiasma in the longer arm, and invariably give rise to rod bivalents, while the remaining four give ring bivalents at metaphase. As the particular bivalents are clearly recognizable at metaphase and anaphase, it is definite that the inversions observed are not contained in these two bivalents. The short and the long rod bivalents are identified with the somatic chromosome types, as chromosome pairs with subterminal and submedian constrictions. This indicates, on the chiasma theory of metaphase association, that ring bivalents are due to the formation and maintenance of at least one chiasma on either side of the spindle fibre attachment. In the subterminally or submedianly constricted chromosomes, the short arms may have paired and formed a chiasma, but the special repulsion force exerted at the centromeres is sufficient to cause their separation before diakinesis, as it is found that the mean frequency of rod bivalents in late diakinesis and metaphase is the same (= 1.7).

Duality of chromatids at second metaphase. In one of the second metaphase plates two of the chromatid ends were observed to be split (Text-fig. 58). The second anaphase chromosomes have been described as bipartite in a number of plants (Koshy, 1934, in Allium, Huskins and Smith, 1935, in Trillium, Sax, 1935, in Rhoeo, and others). It is probable that the split in the chromatids might have occurred in the prometaphase of second division.

Abnormal mutant cell. In an anther locule, showing metaphase I, a mutant cell was observed to have chromosomes thin and elongated like the somatic chromosomes, and nine such bodies could be made out (Text-fig. 61). It is probable that such a condition could have arisen as a result of reduced contraction of the chromosome threads. The presence of more than six bodies indicates failure of some of the chromosomes to pair. Lesley and Frost (1927) have described gene-controlled long-chromosomed meiotic types in *Mathiola*. Probably this may be a case of gene-controlled cell mutation. The cell may probably form a restitution nucleus and may result in a dyad with double the chromosome number in each cell.

Phalaris paradoxa L. var. *praemorsa* Coss. and Dur. ($2n = 14$). Fig. 62 shows the form of bivalents at metaphase. The reduction division seemed to

be regular with the formation of a tetrad of spores. Abnormalities were not observed in the scanty material available for examination.

Phalaris minor Retz. ($2n = 28$). At diakinesis fourteen bivalents were observed with one attached to the large nucleolus with knobs sunk as it were in the vacuole of the nucleolus (Text-fig. 63). In the nucleolar bivalent, crossing-over between two of the four chromatids of parent chromosomes at the chiasma is visible. At metaphase fourteen bivalents are rarely found to show secondary association (Text-fig. 64). The comparative absence of secondary association in recognized tetraploids may be explained as due to: (1) large chromosomes which prevent free movement of the chromosomes enabling them to come together; (2) the ancestral pairs might have become structurally differentiated through a longer period of descent. The low frequency of secondary pairing in cultivated pears, with its high frequency in cultivated apples, is evidence on this point (Moffett, 1934); (3) gene control preventing secondary association. No clear evidence of this has till now been obtained. Examination of wild and cultivated forms of the same species might throw light on the presumed reason (2). Absence of secondary association in polyploid forms with large chromosomes is surely due to the chromosomes offering resistance to movement due to size.

Occasionally a quadrivalent is recognizable in the side views of metaphase (Text-fig. 65 A and B). This is evidence of homologous segments being still present in the ancestral homologues, as this species is definitely known to be a tetraploid by the chromosome number.

One of the bivalents, which evidently has median attachment, is found connected only at one of the ends (Text-fig. 66), showing evidently failure of pairing in one of the arms. If one arm has paired and separated before the other at metaphase it could not be in advance of the attachment region. Another peculiarity is the presence of a large number of bivalents with interstitial chiasma at metaphase, which is perhaps the reason for irregular separation of bivalents at early anaphase (Text-fig. 67). Two cases of interlocking in an association of four have been noted (Text-fig. 68).

Ehrharta erecta Lam. At diakinesis twelve bivalents could be distinguished peripherally in the nucleus, with two bivalents on the nucleolus as in rice (Text-fig. 69). No multivalents were noticed. At first metaphase, secondary association was found to be present between the bivalents, showing that this species is not a simple diploid with basic number (12). Sufficient plates were not available to derive the maximum association. In the fifteen cells examined groups of three were also found to be present (Text-fig. 70 A to E). The evidence is sufficient to show that the basic number is not twelve, as was presumed by Avdulov (1931). He suggested on the small size of the chromosomes, affinities to *Oryza*, which he also considered as having a basic number (12). It is now sufficiently established that the basic number for Oryzeae is five, not only on the evidences of secondary association but also from the examination of the chromosome numbers of other genera belonging to the tribe Oryzeae.



69



A



B



C



D



E

70



A



B



C



D

71



E



F



G



H

71



I



J



K



L

71

TEXT-FIGS. 69-71. Fig. 69. *Ehrharta erecta*. Diakinesis. 12 bivalents, of which 2 are attached to the nucleolus. Fig. 70 (a). *E. erecta*. Polar view. Metaphase I, with 8 bivalents on the periphery and 4 inside. Fig. 70 (b). *E. erecta*. 9 bivalents on the periphery and 3 inside. Fig. 70 (c). *E. erecta*. Secondary association 1(3)+1(2)+7(1). Fig. 70 (d). *E. erecta*.

Ehrharta calycina Sm. Stages earlier than first metaphase were not fixed well for study. Metaphase plates showed twenty-four bivalents. Marked secondary association between bivalents was observed, and of forty-five cells examined, in no cell were they distributed without secondary association. In one or two rare cases there was closer association between two bivalents, indicating multivalent formation. The regular separation at anaphase and the absence of univalents at metaphase indicates that the species is an allotetraploid. Forty-five cells were examined and some of the various types of secondary association observed in polar view are shown in Text-figs. 71 A to K.

Secondary association is an indication of the ancestral homology between the bivalents (Lawrence, 1931) and is dependent on the proximity of such bivalents during diakinesis or at prometaphase, which is partly dependent on chance. Catcheside (1937) concludes from a statistical analysis that secondary pairing is dependent upon the relative positions of bivalents at diakinesis, and the bivalents which happen to lie adjacent at diakinesis and which are capable of secondary pairing are so paired at metaphase. So there is as much chance of maximum association as there is for no association or random distribution. The evidence from the Canti film, of jiggling movements at metaphase, indicates that through such movements chromosomes having a secondary attraction may be brought within each other's sphere of influence and so remain paired. From Table IV no case of absence of association occurred in the cells examined. It has to be inferred, consequently, that a sufficient number of cells has not been examined. It is also probable that a larger number of chromosomes than in the present case reduces greatly the chances of association of all the similar chromosomes. But even from these data, it can be inferred with certainty that the basic number is less than twelve. At early anaphase, repulsion between the secondarily paired bivalents sets in, so that the homologues are no longer clearly paired (Text-fig. 71 L), but since they occupy adjacent positions at telophase and interkinesis, the secondary association reappears in metaphase II.

In two cells the nucleolus persisted at metaphase (Pl. I, Fig. 5; Text-fig. 71 E), and two bivalents were situated on it similar to four chromosomes found on the nucleolus at mitotic prophase. Cases of the nucleolus persisting at meiotic metaphase are rare, and have been reported by Catcheside (1934) in *Brassica* and by Gwynne-Vaughan and Williamson (1933) in many Ascomycetes.

4(2)+4(1). Fig. 70 (e). *E. erecta*. 3(2)+6(1). Fig. 71. *E. calycina*. Polar view of metaphase I, showing various secondary associations. Fig. 71 A. *E. calycina*. 1(5)+1(3)+2(2)+10(1)—5 associations, 1(2) quadrivalent. Fig. 71 B. *E. calycina*. 1(4)+3(3)+2(2)+7(1)—6 associations. Fig. 71 C. *E. calycina*. 2(4)+1(3)+4(2)+5(1)—7 associations. Fig. 71 D. *E. calycina*. 5(3)+2(2)+5(1). Fig. 71 E. *E. calycina*. 3(3)+5(2)+5(1)—8 associations. Fig. 70 F. *E. calycina*. 4(3)+4(2)+4(1)—8 associations. Fig. 71 G. *E. calycina*. 1(6)+1(3)+6(2)+3(1)—8 associations. Fig. 71 H. *E. calycina*. 3(3)+6(2)+3(1)—9 associations. Fig. 71 I. *E. calycina*. 1(3)+9(2)+3(1)—10 associations. Fig. 71 J. *E. calycina*. 2(3)+8(2)+2(1)—10 associations. Fig. 71 K. *E. calycina*. 11(2)+2(1)—11 associations. Fig. 71 L. *E. calycina*. Mid-anaphase showing the disappearance of secondary association.

DISCUSSION

Changes in chromosome complement.

From the general survey of the four genera with the available material the changes in the chromosome complements differentiating species could be inferred as due to any of the following causes: (1) chromosome size, (2) chromosome morphology, (3) chromosome number, (4) structural changes in the chromosome. Uniform change in the size of the chromosome complement as a whole as an expression of genetic control has already been discussed in the early part of this paper. Such changes, bringing about uniform differences in size in nearly related species, may therefore be purely genotypic, and are different from structural changes, which produce differences in the size of individual chromosomes in the complement. In the diploid species of *Phalaris*, the difference between *P. caerulescens* and *P. paradoxa* may be one purely affecting the size of the whole complement, due to genotypic causes.

Changes in the number of chromosomes in related species may be in multiples of a basic set (polyploidy) or may involve the reduplication of some of the chromosomes of a set, both in the diploids and in polyploids (polysomy and secondary polyploidy). From reference to Tischler's (1935-6) and Gaiser's (1930) report of the chromosome numbers for various families and genera in Angiosperms it is safe to say that at least half the species and varieties of flowering plants belong to a polyploid series (Gates, 1924). The origin of polyploids is mainly due to two causes: (1) error in mitosis, either through the failure of the divided chromosomes to separate or through the failure of cell-wall formation, resulting in a tissue which develops into a polyploid plant; (2) disturbances in the sexual process, which leads to the production of polyploid gametes. The nature of the duplicated sets determines the kind of polyploidy. In nature, both allo- and autopolyploidy have played an important part in the evolution of species. In *Phalaris arundinacea*, in the same species races with chromosome sets of fourteen and twenty-eight have been identified. The material examined by Avdulov (1931), Hunter (1934), and by myself showed twenty-eight chromosomes, while Church reports ($n = 7$ and 14).

There are numerous instances of high polyploid races occurring in nature within species. Blackburn (1933) in *Silene ciliata*, Ford (1936) in *Hibiscus*, Erlanson (1929) in *Rosa*, and others.

Müntzing summarizes (1936) fifty-eight instances in which races differing in the number of sets of chromosomes have been discovered. In all these cases the morphological differences between the races were slight, sometimes too slight to merit taxonomic recognition. When these tetraploid races are distinguished by cytological means, it is found that they are on the whole a little larger, considerably stouter, a little longer flowering and hardier, and have a wider distribution as they are able to thrive under a variety of situations. The species *Phalaris arundinacea* is widespread throughout Europe, Asia, and

America, and the cytological examination of this material from different places would indicate the distribution of the diploid and tetraploid races.

On an autopolyploid hypothesis of the origin of these polyploid races it is strange that they breed true. With multivalent formation as a consequence of autopolyploidy, segregation of various aneuploid types would be expected. There are three possible explanations of their regular behaviour: (1) some form of genetic control ensuring bivalent formation, for which there is at present no evidence; (2) that the polyploid form might have risen from the diploid species by hybridization with a second species, followed by selective elimination in the polyploid of all the characters of one parent which distinguish it from the other; (3) genetic and structural differentiation of the duplicated set so that free pairing between all the homologues is prevented.

In addition to the balanced duplication of the whole sets, unequal duplications have been inferred in some genera. Some of the so-called diploids have been found to be secondarily derived from a smaller basic set of chromosomes. Evidence of secondary pairing has brought to light such a condition. In Pomoideae it is assumed that the diploid set of seventeen and its multiples is derived from a basic set of seven (Moffett, 1931). In *Oryza* the diploid set of twelve has been inferred to have been derived from the basic set of five found in other sections of Oryzeae (Nandi, 1936, Sakai, 1935, and Ramanujam, 1938). In *Ehrharta*, although the haploid set is twelve, the evidence of secondary pairing indicates that this has been derived from a smaller basic number, probably five.

Yet another type of change in number relevant in the present studies is the occurrence of a smaller number than the basic set in *Phalaris*. Here the morphological features of the chromosomes indicate the probable nature of the change in the direction of reduction of chromosome numbers. While the *Phalaris* species vary in multiples of seven, two species examined have $n = 6$. One of the species with seven, *Phalaris Lemmoni*, indicates that the nature of the change may be due to the fusion of two chromosomes with subterminal constrictions, giving rise to one medianly or submedianly constricted chromosome. Cases of fusion have been inferred by Gates (1924) in *Drosophila*, Davie (1933) in *Lavatera* and *Gossypium*, Kostoff (1929) in *Nicotiana*, and Lawrence (1931) in *Cardamine pratensis*.

With the present knowledge of the non-fusability of the whole ends of chromosomes, such fusion could arise only by segmental interchange. The two rod-shaped chromosomes with subterminal centromeres could give rise to a single V-shaped chromosome and a small fragment which would get lost, and the chromosome number thus reduced. The efficiency of the fused chromosomes rests on the centromeres being close together. A chromosome with two centromeres situated apart (dicentric chromosome) would fail to survive.

From a comparison of *Phalaris Lemmoni* with *P. caerulescens* and *P. paradoxa*, it is presumed that *P. Lemmoni*, with only two pairs having median

constriction, while all the seven pairs in the latter have median or submedian constrictions, has been evolved by fragmentation of one of the arms, giving rise to subterminally constricted chromosomes. On the hypothesis of Lewitsky (1931) and Levan (1935), that the primitive types have median or submedian constrictions, while with advancing evolution, the chromosomes tend to become subterminally constricted, *Phalaris Lemmoni* should represent a later stage in evolution. So the two types of changes have occurred in the evolution of *Phalaris* species, one in the direction of duplication, giving rise to polyploid races and species, and another in the direction of structural alterations, leading to fragmentation and fusion, resulting in the reduction of chromosome number.

The occurrence of inverted segments in *Anthoxanthum odoratum* and *Phalaris brachystachys* indicates another type of structural change which has been found to result as a consequence of irradiation, as in *Zea Mays* (McClintock, 1931) and in *Drosophila* (Painter and Stone, 1935; Grüneburg, 1935), or of hybridization between different species, as in *Crepis* (Müntzing, 1934), in *Triticum* (Mather, 1935), and in *Lilium* (Richardson, 1936). Evidence of their presence in natural populations has also been found in species of various plants and animals; Smith (1935) in *Trillium*, Upcott (1937) in *Tulipa*, and Darlington (1936b, 1937a) in *Chorthippus* and *Stauroderus* and *Tradescantia*. In *Drosophila* the inversions distinguish natural races (Sturtevant and Dobzhansky, 1936; Koller, 1935).

Inversions may arise from the breakage of a chromosome at the crossing-point of a loop and a different mode of reunion of the end. Reciprocal translocations can also produce inverted arrangements in the middle of segments. If two chromosomes, A B C D and E F G H interchange segments, four new types of chromosomes may result. By the interchange of A and E (1) E B C D and A F G H, and by the interchange of A and H (2) H B C D and E F G A, and if these four new types pair, the configurations will be

$$\begin{array}{c} \text{D C B H-H G F A} \\ | \\ \text{D C B E-E F G A} \end{array}$$

The segment F G is reversed in the opposite chromosomes. Brink and Cooper (1932) suggest that the opposing complexes (middle segments) of *Oenothera* may contain genes in inverted order which might have been derived in the above way. Recently Marquardt (1937) found that the middle segments of *Oenothera* pair straight at pachytene, which is probably due to the continuation of pairing at the homologous end regions. He is of opinion that the differential or middle segment is heterochromatin devoid of genes, but as it is recognized that the middle segments determine the complex differences in *Oenothera*, such an inference is untenable. It is possible that the immediate adjacent regions on both sides of the centromere are heterochromatin and the rest of the middle segment, euchromatin.

These inverted regions have been found to suppress crossing-over. The genetical suppression of crossing-over in certain regions of the X-chromosome

in *Drosophila* has been found to be due to inverted segments (Sturtevant and Dobzhansky, 1936). Even though the chromosomes may pair straight, giving rise to non-homologous association, no crossing-over, evidently, occurs in these regions. Darlington (1936) infers such non-homologous association in the inversions of *Chorthippus* and *Stauroderus*, as not all the cells showed loop pairing—i.e. pairing of the homologous regions in the inversion. The rare occurrence of the bridges and fragments in inversion heterozygotes evidently depends partly upon such an association preventing crossing-over and partly on the length of the inverted region. If the length of the inverted segment is small, the chance of crossing-over is correspondingly reduced. Such a suppression of crossing-over in small inverted segments would result in the group of genes in such a region being inherited as a unit. Any mutations occurring in these regions will tend to be perpetuated, and this will lead to the formation of a new genetically isolated race. The fact that *Phalaris brachystachys*, a wild species, has a small inverted segment present in one chromosome, while the allied species *P. canariensis* does not show evidence of inversions, is significant in this connexion.

SUMMARY

The somatic chromosome numbers of thirteen species representing four genera have been determined in the tribe Phalarideae, and studies have been made of the chromosome morphology.

The tribe is divided into two morphological groups, one comprising the genera *Phalaris*, *Hierochloa*, and *Anthoxanthum*, and the other *Ehrharta*, *Microlaena*, and *Tetrarrhena*. Both taxonomically and cytologically the second group has greater affinities with the Oryzeae than the first group.

The study of mitosis and meiosis leads to the following inferences as regards species differentiation:

1. Genotypic differences controlling the size of the chromosomes have arisen in *Phalaris caerulescens* and *P. paradoxa*.
2. Two types of change have taken place in the evolution of *Phalaris* spp.: (i) duplication of chromosome sets, leading to polyploid races and species, as in *P. tuberosa* and *P. arundinacea*; (ii) structural alterations leading to fragmentation in *P. Lemmoni* and fusion resulting in the diminution of chromosome number in *P. canariensis* and *P. brachystachys*.
3. Segmental interchange coupled with autoploidy in *Anthoxanthum odoratum*.
4. Inversions in *Phalaris brachystachys* and *Anthoxanthum odoratum*.
5. Secondary polyploidy in *Ehrharta*. The changes in the chromosome complement are discussed in relation to the evolution of species.

ACKNOWLEDGEMENTS

It is with great pleasure that I acknowledge my deep indebtedness to Professor R. R. Gates for guidance, help, and criticism in this investigation.

My thanks are due to the Government of Madras for granting me facilities for study.

I am thankful to Mr. C. S. Semmens for the photomicrographs illustrating the paper.

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EXPLANATION OF PLATE

Illustrating Dr. N. Parthasarathy's paper on 'Cytogenetical Studies in Oryzeae and Phalarideae. II. Cytological Studies in Phalarideae'.

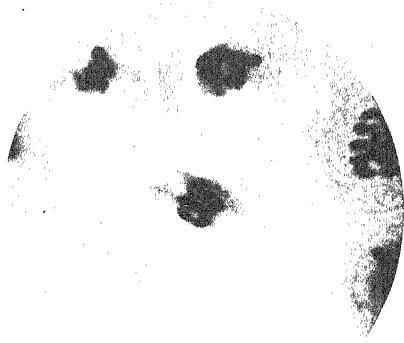
PLATE I

Fig. 1. *Anthoxanthum odoratum*. Side view of metaphase I, showing the centromere in one of the chromosomes (cf. Fig. 32). $\times 2,000$.

Fig. 2. *Phalaris canariensis*. Pachytene, showing the unpaired regions near the attachments to the nucleolus (cf. Fig. 49). $\times 2,000$.

Figs. 3 and 4. *P. brachystachys*. Dicentric chromatid bridge and fragment (Fig. 53). C = $\times 2,500$. D = $\times 2,000$.

Fig. 5. *Ehrharta calycina*. Polar view of metaphase I, showing the persistent nucleolus and secondary association (cf. Fig. 71 E). $\times 3,500$.



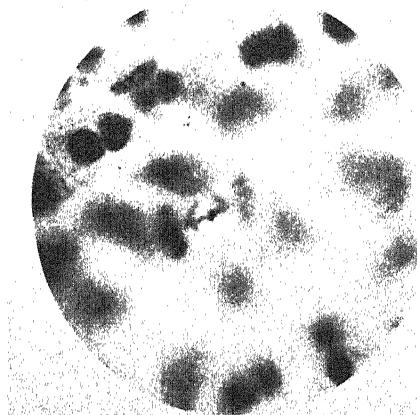
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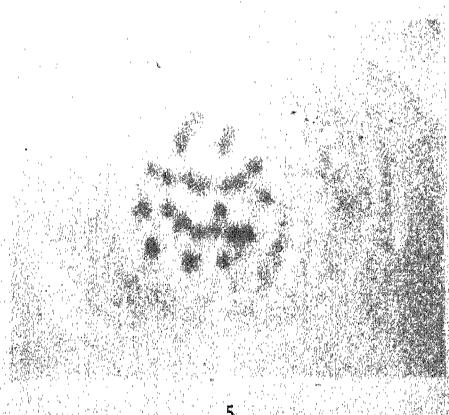
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3



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5

Studies in Tropical Fruits

V. Some Anatomical Aspects of Fruit-fall in Two Tropical Arboreal Plants

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With fourteen Figures in the Text

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I. INTRODUCTION: THE PROBLEM OF FRUIT-FALL

WHEREAS the phenomenon of leaf-fall has been the subject of both anatomical and physiological investigations, the problem of normal fruit-fall appears to have been almost completely neglected. Reference to standard text-books and journals, for example, yields practically no directly relevant data; and so far as the present writer has had access to general botanical literature no foundational observations on this subject have come to hand. Nevertheless, the problem of fruit-fall deserves careful consideration, not only because of its general biological relation to seed dispersal, but also as an important and possibly critical phase in senescence. The phenomenon of fruit-fall is also of interest in the study of the water relationship between the fruit and the plant to which it is attached.

The anatomical aspect of separation of unripe fruits, where fruit-fall is associated with variable water-relations and other factors, has received some consideration. Heinicke (1917), working on the abscission of flowers and partially developed fruits of the apple, detected an absciss-layer 'similar to that which precedes leaf-fall' at the junction of fruit stalk and spur. Coit and

Hodgson (1918), discussing the June 'drop' of navel oranges, relate the premature fall of fruits to abnormal water relations within the plant and ascribe abscission to the 'gelatinization and dissolution of the cell walls resulting in complete separation of cells'. Fawcett (1936) quotes the observations of these authors concerning the colour change in the skin of older, prematurely shed fruits. In these there is a lightening of the green colour, with sometimes a yellow coloration round the apex, several days before separation. Horne (1934), investigating abscission of flowers and immature fruits in the avocado, mentions briefly that the separation of the fruits is due to 'changes in the plant cells' in that plane.

On *a priori* grounds it might perhaps be anticipated that the mechanism operative in the production of leaf-fall, namely, the development of an abscission layer, is also that primarily responsible for the separation of the mature fruit from its parent branch. Again, fruit-fall may be yet a further expression of the various histological and biochemical changes involved in normal ripening and senescence. Other provisional hypotheses could also be advanced. The purpose of the present investigation has therefore been a critical study of certain anatomical aspects of fruit-fall in tropical arboreal plants. For this purpose the mango (*Mangifera indica*) and the avocado (*Persea americana*), have been selected.

As a practical issue, knowledge of the approach to fruit-fall, as an indication of harvesting maturity for commercial storage, has long been recognized as important.

II. MATERIALS

Specimens of *Mangifera indica*, var. Julie, and of *Persea americana*, var. River, were collected from the Agricultural Experiment Station, Trinidad, B.W.I., during the year of 1937. Whenever possible, fresh material was used for sectioning, particularly torn surfaces of freshly fallen fruits, but when material had to be kept for a number of weeks it was preserved in 40–50 per cent. alcohol. Drawings of sections were made by means of a camera lucida, the sections being mounted in 50 per cent. glycerine. The staining reagents principally used were: methylene blue and Schultz's solution for cellulose walls; phloroglucin and hydrochloric acid for lignified walls; concentrated potassium hydroxide and concentrated sulphuric acid for suberized walls; ferric chloride for tannins and ruthenium red for middle lamellae.

III. FRUIT-FALL IN THE MANGO

(a) Stages in fruit development.

Fig. 1 shows the hypogynous, pentamerous flower of the mango in which the gynaecium is reduced to one carpel; the androecium may undergo reduction to one fertile stamen; and the floral axis between the corolla and the androecium is lengthened and thickened, forming a small cushion of tissue. The floral receptacle joins a short pedicel (Fig. 2), the union being marked

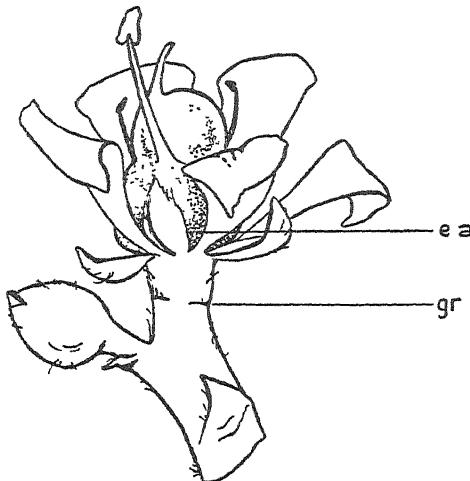


FIG. 1. Mango flower with petals withering (magnified 11 times): *ea*, extension of floral axis; *gr*, circular groove at union of pedicel and receptacle.

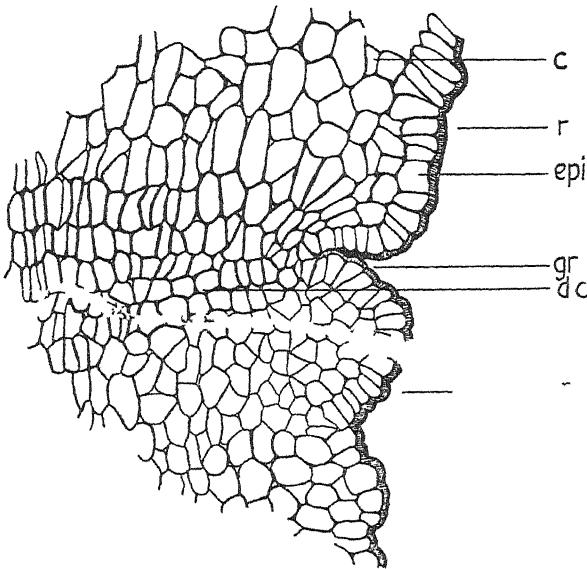


FIG. 2. Mango. L.S. through cortex at union of pedicel and receptacle of flower: *c*, cortex; *r*, receptacle; *epi*, epidermis; *gr*, groove; *dc*, slightly differentiated cells along which fruit later separates from stalk; *p*, pedicel. $\times 162$.

externally by a thin circular groove and internally, in the outer cortex, by two or three irregular layers of small cells, slightly elongated tangentially and with dense contents.

Usually only one fruit (sometimes two or three) develops to full size from each compound racemose inflorescence (terminal or axillary), so that the fruit

stalk eventually consists of part of the main inflorescence stalk and the lateral branch from which the fruit arises, as in Fig. 3. The increasing weight of the growing fruit soon causes it to become pendant on its stalk. As the fruit, a drupe, reaches its maximum size there is a marked increase in growth at the base of the fruit causing raised 'shoulders' surrounding a small 'island' of tissue attached to the stalk.

The original cushion between the corolla and the androecium is apparent as a scarred narrow band and the groove at the base of the floral receptacle is clearly marked an eighth to a quarter of an inch beyond.

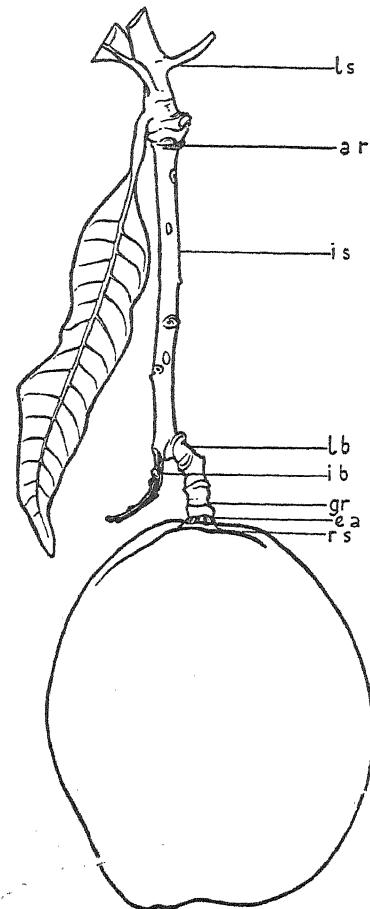
As a rule, mangoes with raised 'shoulders' will ripen at tropical temperature in two to four days after picking, but if left on the tree several weeks may elapse before normal fruit-fall takes place. Using fruits of this maturity series of transverse sections were made through the leafy stems and fruit stalks arising from them. All showed a central xylem cylinder enclosing a pith and a cylinder of phloem surrounded by a cortex and epidermis. The leafy stem was characterized by a predominance of xylem over phloem tissue, whereas in the fruit stalk, approximately equal quantities of phloem and xylem were present in the region farthest from the fruit, changing to a higher proportion of phloem to xylem near the fruit. Beyond the grooved region near the fruit the amount of xylem was much decreased, the cylinder breaking up into an irregular ring of strands separated by wide areas of parenchyma, and into twisted branching strands in proximity to the latex canals in the cortex (Fig. 4).

Here the latex canals were larger than in the stalk; the cortical cells possessed thickened cellulose walls and there was an absence of the lignified cells found elsewhere in the pith.

FIG. 3. Full green mango fruit: *l s*, leafy stem; *a r*, abscission region; *i s*, inflorescence stalk; *l b*, lateral fruit-bearing branch; *i b*, withered branch; *gr*, groove; *e a*, extension of floral axis; *r s*, raised shoulders of fruit. $\frac{1}{2}$ nat. size.

(b) Ripening and fruit-fall.

The first external sign of ripening in the Julie mango on the tree is a softening of the fruit pulp. Soon after this the fruit separates from its stalk, rotting of



this variety on the tree being seldom observed. Anatomical studies of such ripening attached mangoes were made, fruit stalks being examined for evidence of drying out, absciss cork formation or other histological changes which would result in curtailing or inhibiting access of water from the leafy stem to the fruit.

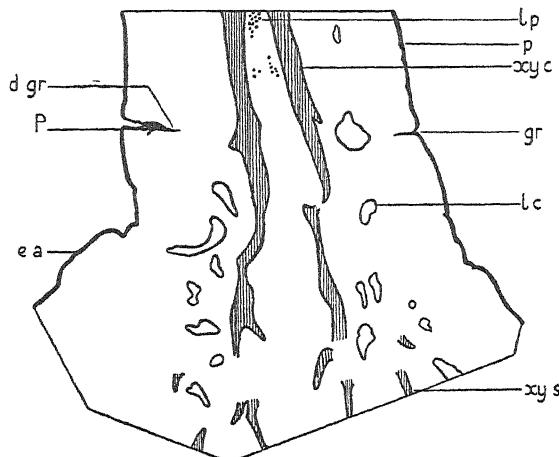


FIG. 4. Mango. Diagram of L.S. through stalk of fruit in region of groove: *l.p.*, lignified pith; *p*, pedicel; *xy.c*, xylem cylinder; *gr*, groove; *l.c*, latex canal in cortex; *xy.s*, xylem strand; *d.gr*, deepening of groove along middle lamellae of cortical cells; *P*, see Fig. 5; *e.a*, extension of floral axis.

Only slight evidence of drying out in the fruit stalk was obtained, a few vessels showing small tyloses. An increase in tannin crystals (where before tannins had been present chiefly in the dissolved form) was observed in the cortex near the groove. Meristematic activity of the surrounding cells caused a blocking of the latex canals in the same region.

Careful examination showed that the important histological changes associated with fruit-fall were to be observed in the cells of the flesh adjacent to the groove. In these the walls had become swollen and mucilaginous, the inter-cellular spaces being thereby occluded or obscured. These changes, which are typical of the ripening of the flesh, were traced as extending progressively into the fruit stalk towards the groove where, also, the collenchymatous cell-walls had become slightly swollen and gelatinous so that several of the air spaces were scarcely detectable. In some fruits the grooves showed signs of deepening, as in Fig. 4, due to the break-down and splitting along the middle lamella of adjacent cortical cells.¹

Immediately after fruit-fall, in fruits which had been marked and kept under

¹ The observations of Coit and Hodgson (1918) concerning skin-colour change and dissolution of middle lamellae along the plane of separation in older navel oranges, prematurely shed, are of interest in that they suggest that fruits separating even at this early stage exhibit some of the features of ripening associated with normal fruit-fall.

close observation, further investigations were made. It was found that the fruit had separated with a fairly clean break in the plane of the groove and that softening of the fruit stalk to a depth of about 1 mm. from the torn end had taken place. In longitudinal sections of the freshly torn surfaces of fruit and stalk it was seen that the cleavage had occurred along the middle lamellae of the cells, the cell walls and contents remaining undamaged, Fig. 5. There was no evidence of suberization nor of the formation of an abscission cambium.

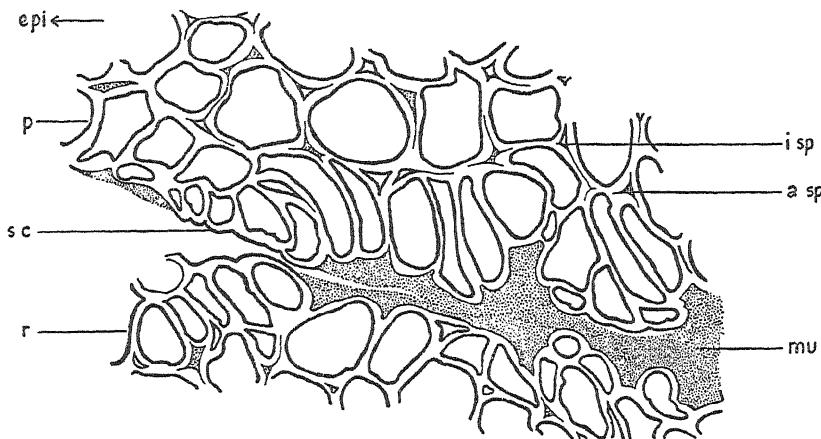


FIG. 5. L.S. through stalk cortex of ripe attached mango in region of groove, at Position P of Fig. 4: *i sp*, intercellular space; *a sp*, air space obscured by swollen and gelatinous walls; *mu*, mucilaginous substance filling up space left by separated cells; *epi*, arrow pointing towards epidermis; *p*, pedicel; *s c*, undamaged surfaces of cells separated by break-down of middle lamellae, mucilaginous substance having disappeared; *r*, receptacle. $\times 276$.

(c) Abscission of the fruit stalk.

In the nearly ripe but still attached mango the stalk between the fruit and leafy twig is firm and greenish in colour, sometimes pigmented. Although such stalks, as ripening proceeds, show progressively less and less exudation of latex on breaking or cutting external signs of desiccation have not been observed. On the other hand, desiccation is well marked in the non-fruit bearing branches of the inflorescence.

Once the fruit has fallen, drying out begins in the small fruit stalk and gradually extends back to the leafy shoot (Fig. 6). Evidence of this is to be seen in the yellowing and wrinkling of the stem which eventually becomes brown and hard. When quite withered the branch will snap off easily at its junction with the leafy twig, although under natural conditions it remains attached to the plant for a considerable period of time. This failure to shed rapidly the withering and decaying stalks may have an important bearing on the harbouring of certain pathogens known to be associated with both die-back and fruit diseases.

Coincident with these developments internal changes associated with water

losses occur. The epidermis becomes folded and wrinkled, the thin-walled cells of cortex and phloem become shrunken and compressed, and the latex canals and lignified tissues distorted. The latex dries up and in the pith meristematic activity leads to a blocking of the canals, while phellogens developing in the cortex cut off old injured parts. The tissues in general become discoloured, tannins are found in the protoxylem, and tyloses are everywhere abundant in the xylem. Tyloses have also been observed in some vessels of the leafy stem supporting the fruit.

Only when the withering and drying of the fruit stalk is well advanced does a cambium arise at its junction with the leafy twig, when rows of lignified-suberized cells may be observed, Figs. 7 and 8. This is the only abscission layer formed in the fruit stalk.

IV. FRUIT-FALL IN THE AVOCADO

(a) Stages in fruit development.

The numerous small flowers of the avocado pear are borne on a terminal or axillary inflorescence. They are trimerous with two perianth and four staminal whorls arising from the rim of the slightly perigynous receptacle. The ovary is unilocular with a single pendulous ovule. The union between the pedicel and floral receptacle is marked by a circular indentation but the obvious groove observed in the mango is not present. As in the mango, only a few pendant drupes develop from each inflorescence. In Fig. 9 the attachment of an avocado to its stalk and the relation of the latter to the leafy branch are shown.

Towards maturity rapid localized growth in the fruit, in the region of its attachment to the stalk, results in the formation of a circular pit at the base of which the stalk is attached, as in Fig. 10. Just above this plane of insertion the pith cells in the stalk are lignified. In this region, also, the xylem cylinder separates into the thin strands that supply the fruit.

(b) Ripening and fruit-fall.

As in the mango, the avocado undergoes only slight softening before it falls. The fruit stalk of the newly fallen fruit shows little or no development of tyloses, nor is an abscission cambium visible at the junction with the leafy twig.

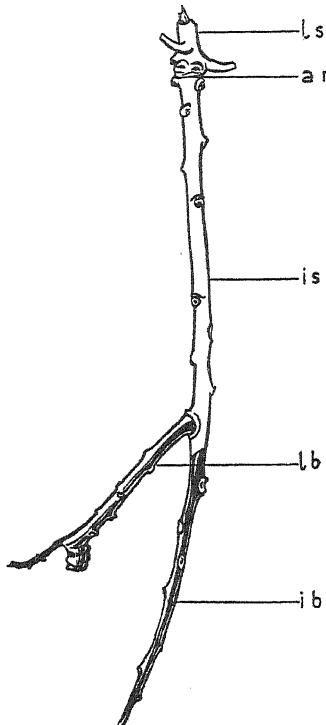


FIG 6. Mango. Fruit stalk showing desiccation: *l s*, supporting leafy stem; *ar*, abscission region; *i s*, inflorescence stalk; *lb*, lateral fruit-bearing branch showing signs of drying out, end torn in plane of groove; *ib*, withered branch. $\times \frac{1}{2}$.

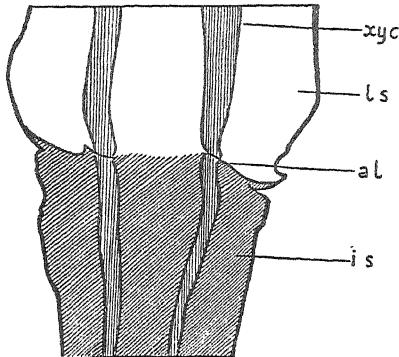


FIG. 7. Mango. Diagram of stem L.S. through junction of leafy twig with much desiccated fruit stalk. *xyc*, xylem cylinder; *l s*, leafy stem; *al*, abscission layer; *i s*, withered fruit stalk.

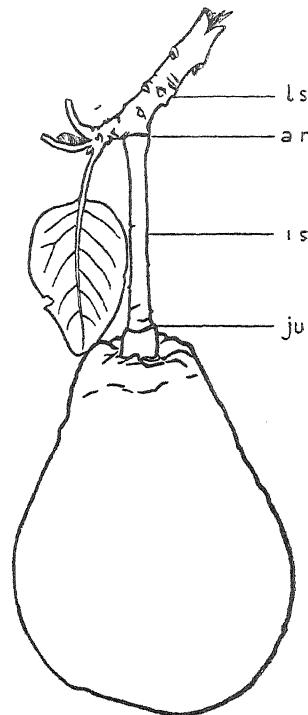


FIG. 9. Full green avocado pear fruit: *l s*, leafy stem; *ar*, abscission region; *i s*, fruit stalk; *ju*, junction between pedicel and floral receptacle. $\times \frac{1}{2}$.

FIG. 9.

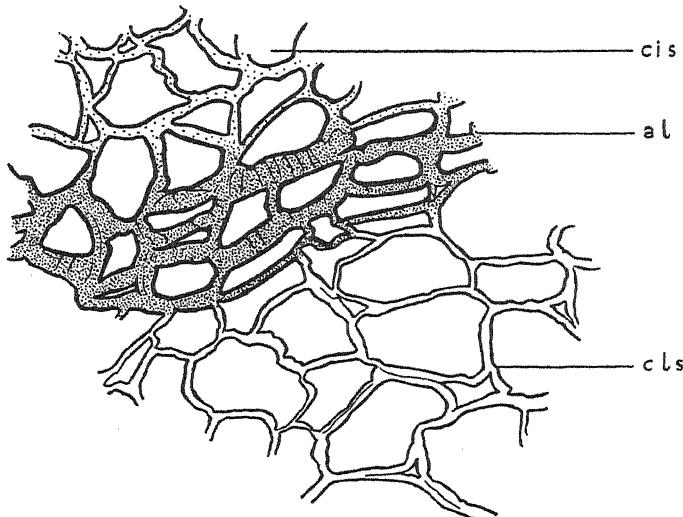


FIG. 8. Mango. L.S. through stem cortex at junction of leafy twig with withered fruit stalk: *cis*, slightly ligni-suberized cortical cells of fruit stalk; *al*, thick ligni-suberized-walled cells of abscission layer; *cls*, cellulose-walled cortical cells of leafy twig. $\times 276$.

During the ripening of the fruit pulp the parenchymatous cells tend to become spherical and may be easily separated, due to the break-down of the middle lamellae. The thin walls, however, undergo little swelling, the inter-

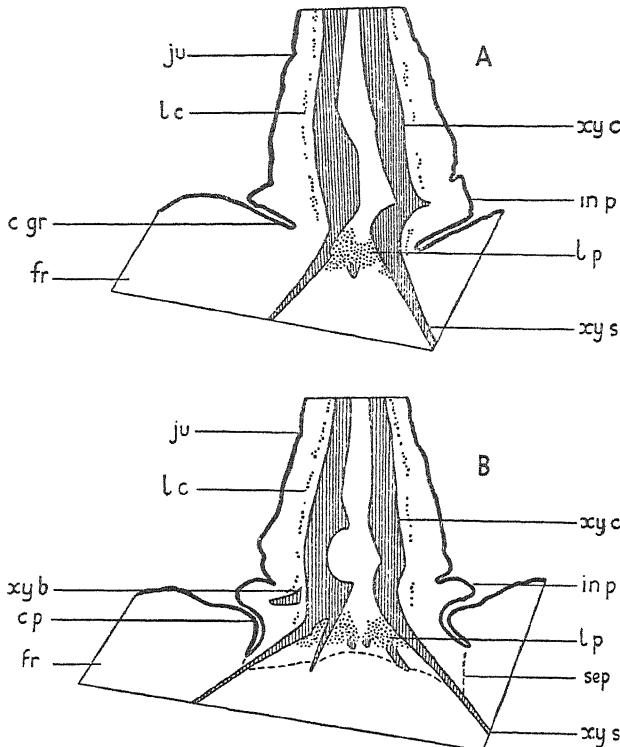


FIG. 10. Avocado pear. Diagrams of L.S. through regions of fruit stalk insertion in (A) young fruit and (B) full green fruit: *ju*, junction between pedicel and receptacle; *lc*, lignified cortex; *c gr*, circular groove where young fruit is attached to stalk; *fr*, fruit; *xy c*, xylem cylinder; *in p*, region of insertion of perianth and androecium; *lp*, lignified pith; *xy s*, xylem strand supplying fruit pulp; *xy b*, xylem branch supplying perianth, &c.; *cp*, circular pit at base of which mature fruit is attached to stalk; *sep*, dotted line indicates region of separation when fruit falls from stalk.

cellular spaces being readily apparent. These features are also characteristic of the cells in the region where the fruit separates from its stalk (Fig. 11). The fall of the avocado therefore does not involve injury to any cell walls, cleavage taking place along the middle lamellae. In all the specimens examined the plane of separation was observed to extend across the stalk by way of the parenchyma lying immediately below the lignified pith cells shown in Fig. 10.

(c) *Abscission of the fruit stalk.*

Subsequent to fruit-fall, the fruit stalks exhibit changes (Fig. 12) similar to those observed in the mango. These, however, are much more rapid,

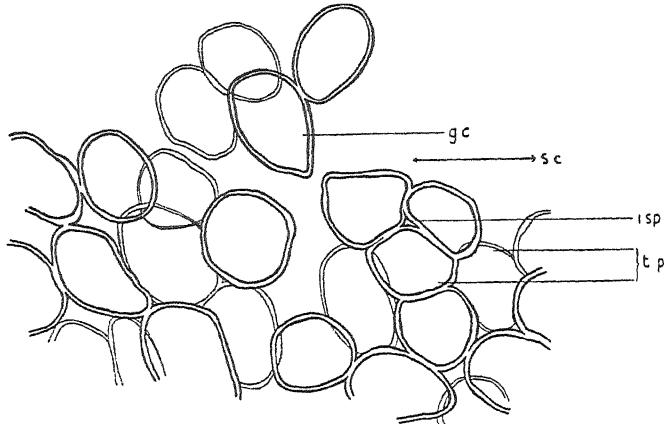


FIG. 11. Avocado pear. L.S. through cortex of stalk end recently attached to ripe fruit: *g c*, group of cells recently attached to stalk end; *sc*, surface at end of stalk composed of undamaged cells separated from cells of fruit by break-down of middle lamellae; *isp*, inter-cellular space; *tp*, cells seen in two planes. $\times 184$.

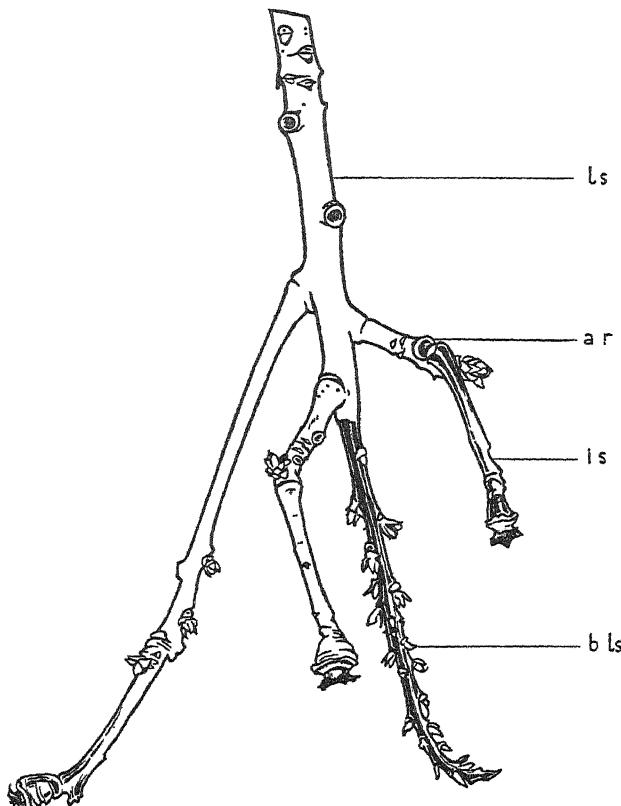


FIG. 12. Avocado pear. Fruit-bearing branch showing desiccation: *ls*, leafy stem; *ar*, abscission region; *is*, dried fruit stalk nearly detached; *b ls*, withered branch of leafy stem with vegetative buds. $\times \frac{1}{2}$.

abscission of the stalks taking place a few weeks after fruit fall. Withdrawal of water resulting in wrinkling and a change of colour through yellow to brown renders the stalk increasingly dry and brittle.

Internally, the xylem becomes blocked with tyloses, soft-walled cells become compressed and discoloured, while an abscission layer, rather more extensive than in the mango, is formed cutting off the stalk from its supporting leafy twig (Figs. 13 and 14).

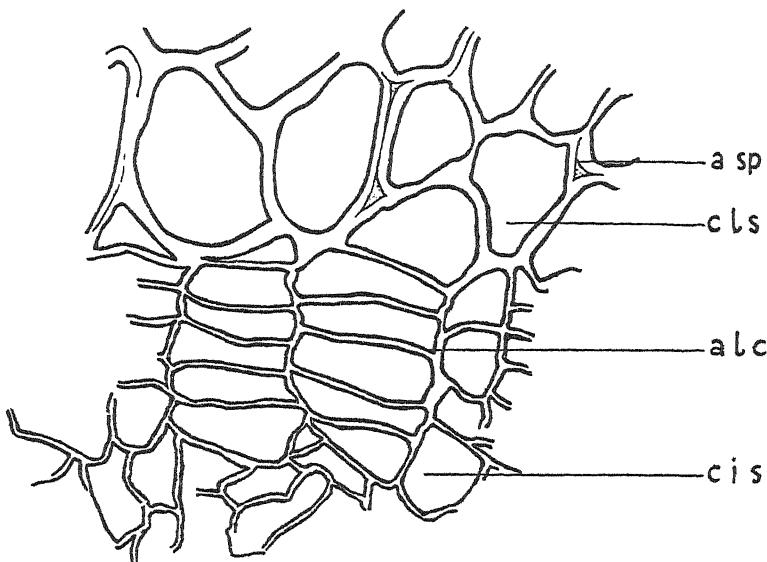


FIG. 13. Avocado pear. L.S. through scar on stem left by recently fallen stalk: *a sp*, air space obscured by gelatinous walls; *cls*, cellulose-walled cortical cells of leafy twig; *alc*, abscission layer cambium slightly suberized; *cis*, ligni-suberized and distorted cortex of fruit stalk. $\times 368$.

V. DISCUSSION AND CONCLUSIONS

From the anatomical data submitted above certain definite conclusions regarding normal fruit-fall in the mango and avocado have been obtained. Whereas shedding of the *fruit stalk* is comparable with leaf-fall, being preceded by the formation of an abscission layer, separation of the fruits from their supporting stalks is the result of histological and biochemical changes of the same kind as those observed during the ripening of the fruit flesh. Moreover, these changes occur some considerable time prior to the development of a stalk abscission layer. The phase of ripening involving dissolution of the middle lamellae of cells at the base of the fruit is primarily responsible for the shedding of the fruit, the weight of the fruit and wind action assisting in this process. It has been shown (Wardlaw and Leonard, 1936) that ripening in the mango proceeds from the stone outwards to the skin. Fruit-fall is therefore seen to take its place as a normal phase in the progressive softening and

disintegration of tissues during the later stages of maturation. The observation has also been made that the position of the plane of separation is pre-determined by morphological and anatomical features such as the juxtaposition of lignified and cellulose-walled tissues, grooves on the stalks, &c.

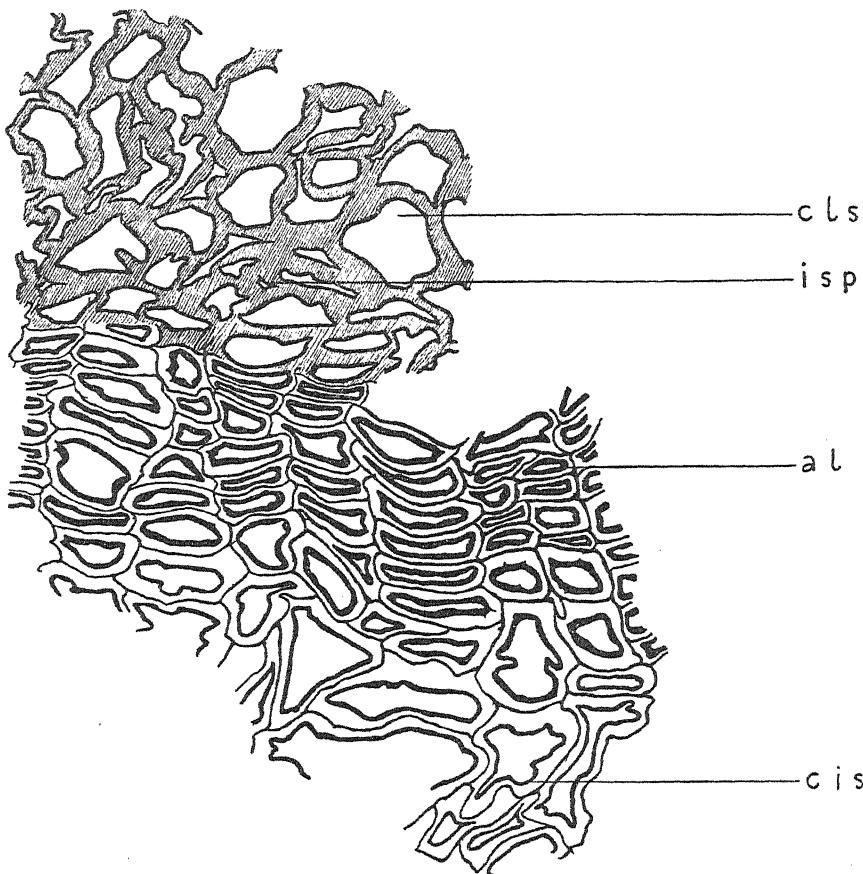


FIG. 14. Avocado pear. L.S. through old scar left by stalk: *c ls*, discoloured and distorted cortical cells of leafy stem; *i sp*, intercellular space; *a l*, ligni-suberized abscission layer; *c is*, distorted cells of fruit stalk cortex, intercellular spaces being obscured by thickened cell walls. $\times 368$.

When mango and avocado trees are not subjected to marked variation in water-supply, which is liable to cause premature fruit-fall, the relatively slow ripening drift of the attached fruits apparently continues undisturbed until an advanced stage (approximately eating-ripe) is reached. The view is held that during this period water relations, as they apply to the fruit as a whole and to its component tissues which are undergoing maturation, are of great importance. The relevant problems are undoubtedly of a considerable complexity and will require detailed investigation along several different lines. During

the progress of the present work the possibilities that the initiation of ripening of fruit on the tree and that normal fruit-fall were related to a curtailment of water-supply were considered. Anatomical observations, however, have not so far yielded data supporting this view; that there is no marked limitation of water-supply prior to fruit-fall is suggested by the negligible development of tyloses, blocking of vessels, &c.

SUMMARY

1. The anatomical changes associated with normal fruit-fall are described for the mango (*Mangifera indica*) and avocado (*Persea americana*).
2. Whereas shedding of the fruit stalk is accompanied by the development of an abscission layer as in leaf-fall, the separation of the fruit from its stalk is primarily due to the dissolution of the middle lamellae in the tissues at the base of the fruit.
3. Fruit-fall is therefore a normal phase in the progressive softening and disintegration of tissues during the later stages of ripening.
4. The position of the plane of separation is predetermined both by morphological and anatomical features.
5. Absciss cork formation is evident only at the junction of fruit stalk and leafy stem and develops some time after fruit-fall has taken place.

ACKNOWLEDGEMENT

My thanks are due to Dr. C. W. Wardlaw for his constructive criticism and for the opportunity afforded me of carrying out this investigation at the Low Temperature Research Station, Trinidad, B.W.I.

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The Sexual Reproduction of *Ranunculus Ficaria*

BY

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With fifteen Figures in the Text

INTRODUCTION

HERE are frequent references in the literature to the fact that *Ranunculus Ficaria* L. very seldom sets viable seed. The reasons for this have been investigated especially by Souèges (1913), Kindler (1914), Loschnigg (1925), and Wolter (1933). These authors review previous investigations and theories concerning the possible causes of the reduced sexual fertility of the species, so there is no need to record in detail the numerous papers which were published before those cited above. The present investigation was undertaken in continuation of those concerning the life-history of *R. Ficaria* by Marsden-Jones (1935), and my studies (1936, 1938) of the morphological nature of the single cotyledon and the mode of development of the aerial and subterranean tubercles. Of the previous investigators mentioned above, Kindler, Loschnigg, and Wolter appear to have worked almost exclusively with material from various parts of central Europe, where, it seems evident from their papers, the strains of *R. Ficaria* are particularly sterile and are always provided with axillary tubercles above soil level. Wolter, however, records that he received material from Exeter (England) which was far more productive of viable seed than were the local strains. The significance of this observation will be seen from what follows. All these authors are agreed that the lack of viable seed is primarily due to various types of disorganization of the embryo-sac and nucellus, of very much the same types as I have observed and fully describe below. The workers in central Europe also believe that failure of the pollen to germinate is partly responsible for the lack of viable seed. I have not investigated this possibility to any great extent, but the frequency with which various forms of disorganization occur in ovaries, even when well developed pollen tubes are present, rather suggests that lack of viable pollen is a relatively unimportant cause of sterility in English strains of *R. Ficaria*. Kindler and Loschnigg express the opinion that the causes of the break-down of the gynoecium are inherent in the plants themselves, being so fixed that the percentage of viable seed cannot be modified by change of environment. Wolter, however, when referring to this view, also records that Dostal carried out transplant experiments which led him to suppose that the fertility of *R. Ficaria* is capable

of being partly controlled by change of environment. It may be mentioned in this connexion that in a few sand-culture experiments carried out at Kew, forms of *R. Ficaria* with and without aerial tubercles respectively failed to exhibit any change of fertility corresponding to variations in the mineral composition of the culture fluids with which they were watered. Likewise the balance of evidence from the Continent seems to favour the view that sterility is a relatively fixed character.

Marsden-Jones (*loc. cit.*), who worked with British material, records the existence of forms of *R. Ficaria* which produce relatively large and relatively small numbers of viable seeds respectively. From the relatively fertile forms aerial tubercles in the leaf axils are absent altogether, but they are present in those in which the production of viable seed has been considerably reduced. In his paper Marsden-Jones refers to cytological investigations which showed that the forms with and without aerial tubercles respectively are genetically distinct, and he therefore proposed the varietal name *bulbifera* to include all plants bearing axillary tubercles above soil level. This is a distinct departure from the attitude of those who worked with material from central Europe, all of whom agree that aerial tubercles are universally present, although the number on individual plants and in different localities was found to vary considerably.

My own field observations in parts of Surrey, Middlesex, Buckinghamshire, Wiltshire, Dorset, Devonshire, Somerset, and Hereford have fully confirmed the existence of distinct forms of *R. Ficaria* with and without aerial tubercles respectively, and those without invariably produce a higher proportion of viable seed than do those with aerial tubercles. The relative frequency of these forms in different parts of the country is interesting. No detailed survey of the country as a whole has yet been completed, so that the observations here recorded must be regarded only as a contribution to the subject of distribution. In Devonshire, where somewhat extensive surveys, especially near Exmouth, Budleigh Salterton, and Sidmouth, have been made during the past few years, no plants with aerial tubercles have been observed; and in these districts a remarkably high proportion of apparently viable seed is produced. Likewise in other parts of Devonshire, also near Minehead and on or near parts of the Quantock Hills in Somerset, no plants with aerial tubercles have been found, although the searches made in these districts were not sufficiently complete to show that forms with aerial tubercles are absent. A notable exception was in a garden at Stoke Canon, near Exeter, where plants with aerial tubercles were more frequent than those without, although in wild habitats near the village only forms without aerial tubercles were seen. The only other observations made in the West of England were in the neighbourhood of Colwall, near Malvern. In the district immediately round the village both forms were present, but plants without aerial tubercles appeared to be dominant. From Lyme Regis, in the extreme west of Dorset, a form of *R. Ficaria* with aerial tubercles was received from Dr. A. Arber, but otherwise no information from this locality is available. In the more eastern part of the

county, however, notably round Wareham and in some of the villages just south of Blandford, fertile, free flowering races of *R. Ficaria* occur, but relatively sterile forms with aerial tubercles are also fairly frequent. Observations made by Marsden-Jones in the Devizes district of Wiltshire show that forms with and without aerial tubercles are both fairly frequent. In still more eastern parts of the country, for instance in Middlesex, parts of Surrey, and in Buckinghamshire both kinds occur, but forms with are generally more frequent than those without aerial tubercles. The floor of some of the beech woods on the Chiltern hills is frequently covered in spring-time with a pure stand of *R. Ficaria* with scarcely any flowers at all, whilst those that are present produce little or no apparently viable seed. These sterile forms invariably bear aerial tubercles.

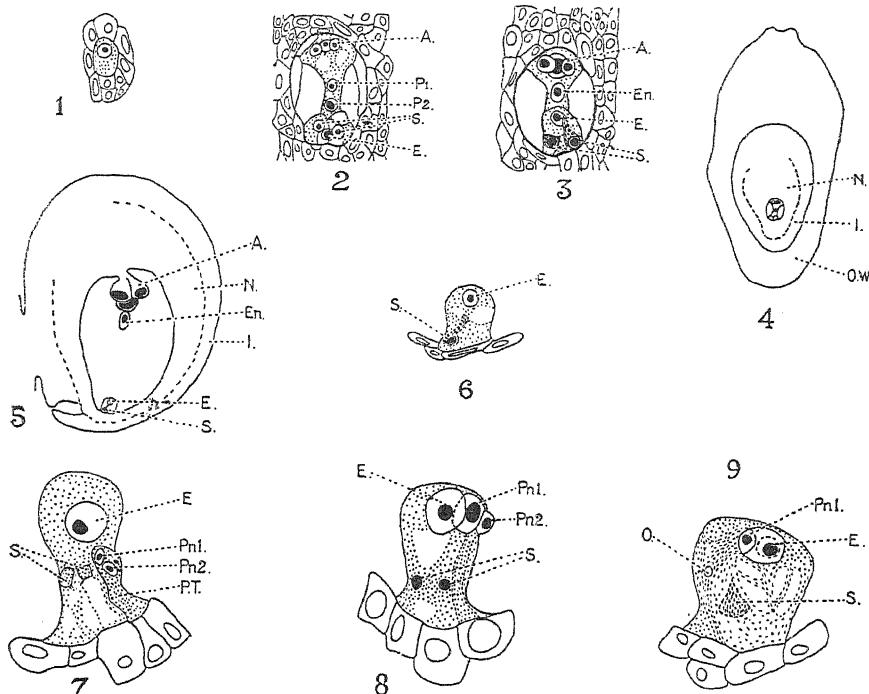
In making this admittedly very incomplete survey I have been very much impressed by the undoubtedly dominance of sexually productive forms in the south-west of the country, and their relative infrequency in the Buckinghamshire and Middlesex districts. Forms with aerial tubercles appear at least to be dominant in central Europe. It is also interesting to note that the fertile material which Wolter obtained from England came from the Exeter district, where my own observations have shown that the local races produce a high proportion of viable seed. The species thus presents problems of considerable interest. The work cited by Marsden-Jones (*loc. cit.*) suggests that the forms with aerial tubercles are cytologically and genetically distinct from those without them. In a previous communication (1938) I suggested that the production of aerial tubercles, which consist morphologically of adventitious roots bearing one or more buds, may be governed by the action of a hormone-like substance. Whether this surmise is correct remains to be seen, but it is at least significant that the production of adventitious roots in other plants appears to be controlled by the so-called phytohormones. The mode of origin and distribution of genetically distinct forms within such a well-defined species as *R. Ficaria* is a problem that may well tax the ingenuity of physiologists, geneticists, and ecologists for some time to come. Attention has been drawn by Schwarzenbach (1922) to the somewhat similar problems presented by *Cardamine bulbifera*, where, as in *R. Ficaria*, the development of asexual reproductive bodies has been accompanied by loss of the capacity to reproduce by sexual means.

STRUCTURE OF THE NORMAL EMBRYO-SAC AND FERTILIZATION

Although much has been written concerning the embryology of the Ranunculaceae, very little appears to be known concerning the actual process of fertilization in this family. Coulter (1898), Thomas (1900), Guignard (1901), and Souèges (1910, 1911), however, have described fertilization in one or more species of Ranunculaceae. In spite of the extensive researches on *R. Ficaria* carried out by the various authors who have already been mentioned, it is interesting to note that none of them has observed actual fertilization in

this species. The most complete account of the development of the embryo-sac of *R. Ficaria* is that given by Souèges (1913), but he was unable to include particulars of fertilization because so many of the ovules become disorganized. He says that the hypodermal archesporial cell divides to form two daughter cells, the lower one of which then divides again to form two megasporangia. Soon afterwards the lower megasporangium develops into the embryo-sac. I have not been able to confirm the above course of events in the material available, as none of it was examined at quite the right stage. Nor was it possible to determine exactly the course of events between the uninucleate stage of the embryo-sac and its maturity, although various stages in the process of fertilization were observed in material from Devonshire and Somerset. The embryo-sac mother-cell can generally be distinguished from its neighbours in the nucellus by its larger size (Fig. 1). It seems probable that the embryo-sac reaches the 8-nucleate stage in the manner that is usual in other members of the Ranunculaceae, although the first well-defined stage to be recognized in my material was as in Fig. 2, at the upper end of which three nuclei (A) destined to become the antipodal cells may be discerned, whilst two polar nuclei (P_1 and P_2) are about to fuse to form a large endosperm nucleus at the centre of the sac. At the micropylar end of the sac there are three nuclei of equal size, of which two are the young synergids (s) and the third is the egg cell (E). At a slightly later stage (Fig. 3) the antipodal cells have increased in size, and there is a large central endosperm nucleus (en.). The egg cell (E) has also become enlarged, whilst the two synergids have not increased in size to the same extent. Very frequently there is a particularly dense patch of cytoplasm between the nucleus of one of the synergids and that of the egg cell. This is to be seen also in Fig. 6, which shows one of the synergids (s) of an older embryo-sac in relation to the egg cell. The embryo-sac reaches the stage of development shown in Fig. 3 before the flower is fully opened, and frequently remains like this until the time of fertilization, although the antipodal cells, as in so many Ranunculaceae, become considerably enlarged and are then very conspicuous (Fig. 5). Fig. 4 shows the embryo-sac of Fig. 3 at a lower magnification, so that its position in relation to the nucellus (N) surrounded by the integument (I) and the ovary wall (O.W) may be seen. The pollen tubes in fertile material develop very rapidly when once the grains have alighted on the stigma, growing downwards through the wall of the ovary. It is easy to follow their passage for a certain distance because chemical changes cause the tissues through which they are passing to stain more readily. As they approach the micropyle, however, the pollen tubes cannot readily be detected, so that the latter part of their course was not observed. Nevertheless, careful examination of ovules in which the pollen tubes could be seen to have penetrated deeply, sometimes revealed a vesicle containing two nuclei (Pn_1 and Pn_2) within the embryo-sac in the region of the egg cell (Fig. 7 P.T.). This undoubtedly represents the swollen tip of the pollen tube. The vestigial remains of the synergids (Fig. 7, s) were sometimes visible as well. Guignard (loc. cit.), in his account of fertilization in the

Ranunculaceae, says that the contents of the pollen tube are generally discharged within one of the synergids, although he mentions that direct contact between the pollen tube and the egg cell is sometimes made without the intervention of a synergid. The discharge of the pollen tube into a synergid was



Figs. 1-9. Fig. 1. Embryo-sac mother-cell, surrounded by cells of nucellus. $\times 220$. Fig. 2. Embryo-sac nearly mature. $\times 220$. Fig. 3. Embryo-sac at slightly older stage than in Fig. 2. $\times 220$. Fig. 4. L.S. ovary showing embryo-sac of Fig. 3 at a lower magnification. $\times 48$. Fig. 5. Mature embryo-sac ready for fertilization. $\times 48$. Fig. 6. Egg apparatus of Fig. 5 at a higher magnification. $\times 220$. Figs. 7-9. Egg cells showing three consecutive stages of fertilization. $\times 440$. A. Antipodal. E. Egg cell. En. Endosperm nucleus. I. Integument. N. Nucellus. O. Unidentate nuclear body. ow. Ovary wall. p₁ and p₂. Polar nuclei. Pn₁ and Pn₂. Pollen tube nuclei. P. T. Pollen tube vesicle. s. Synergids.

not observed in *R. Ficaria*. In fact, one or both synergids were generally in a state of disorganization at the time of fertilization. Slightly later stages than that in Fig. 7 may be seen in Figs. 8 and 9. In Fig. 8 one of the pollen nuclei is about to fuse with that of the egg cell, whilst the second is about to be set free into the cavity of the embryo-sac. The remains of the pollen tube may be discerned below the pollen tube nuclei, and vestiges of the synergids (s) are also to be seen. At the stage in fig. 9 one pollen tube nucleus has actually fused with that of the egg cell. A dense part of the cytoplasm is all that remains of the synergids. A small darkly stained body of uncertain nature (o) was also observed.

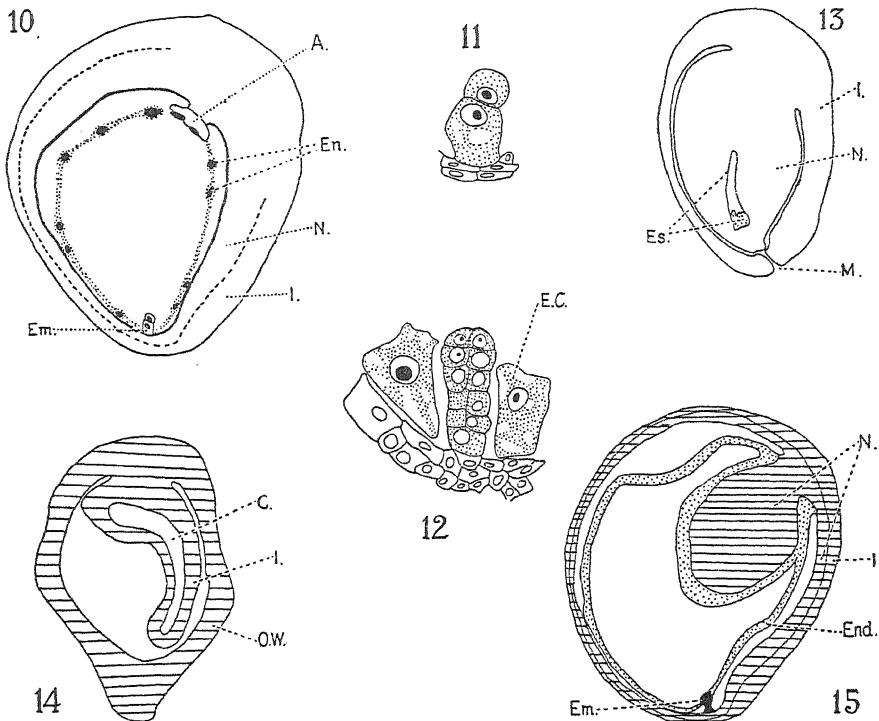
I was unable to determine what became of the second nucleus from the pollen tube that it liberated into the embryo-sac, but it apparently becomes disorganized. One would expect, by analogy with what happens in other plants, that it would fuse with the central endosperm nucleus as a preliminary to the further development of the endosperm, but this was never seen to occur. The central endosperm nucleus generally passes towards one side of the embryo-sac at a time when the short length or absence of pollen tubes showed that fertilization could not possibly have taken place. It is clearly evident that fusion with a nucleus from the pollen tube is unnecessary to stimulate its further development. *R. Ficaria* thus conforms with certain other species of Ranunculaceae in which Coulter (loc. cit.) demonstrated that fertilization is not a necessary preliminary to endosperm development. Having reached one side of the embryo-sac the endosperm nucleus divides repeatedly, giving rise to a number of small nuclei which line the interior of the sac (Fig. 10). As the ovule matures the initial layer of endosperm increases in bulk, becomes cellular, and gradually fills the cavity of the embryo-sac, which by then has become considerably enlarged. In Fig. 10 the fertilized egg has divided to form a two-celled embryo (Em), which is drawn at a higher magnification in Fig. 11. A more mature embryo with two adjacent endosperm cells (Ec) is shown in fig. 12.

ANOMALOUS EMBRYO-SACS AND NUCELLI

The above course of events seems to be usual in the strains of *R. Ficaria* from the south-west of England, and probably also in some of the sexually fertile strains from other parts of the country. In the ovaries of the more sterile races, however, especially when aerial tubercles are present, various forms of abnormality occur. The young ovaries of these generally appear to be normal, but as they grow older one or more of the cells within the embryo-sac become disorganized. Sometimes only one or both of the synergids are missing from otherwise complete embryo-sacs, or, in other instances the endosperm nucleus is absent. The antipodals are more resistant to decay, and could frequently be recognized when the entire egg apparatus and endosperm nucleus had disappeared. Ovaries in which the full complement of embryo-sac nuclei are never formed were also seen, in which case the embryo-sac is reduced to a slit-shaped cavity as es in Fig. 13. At the micropylar end of the particular embryo-sac shown in this figure a certain amount of granular material is present, but in other instances the sacs were devoid of contents. In some ovaries no embryo-sacs appeared to develop at all.

By far the commonest form of disorganization, however, was due to the break-down of the nucellus itself, the collapse usually starting in the cells immediately around the embryo-sac and spreading outwards through the tissues, so that eventually a hollow cavity marked the position in which the nucellus should have been situated, the integument remaining intact (c in Fig. 14). It was quite common for the cavity of the ovary to become enlarged

even when the nucellus had become disorganized, so that the mature ovary consisted for the most part of a hollow sphere with the remains of the integument on one side. An ovary of the type illustrated in Fig. 14 would probably become like this at a later stage of development. Another abnormality,



Figs. 10-15. Fig. 10. Enlarged embryo-sac and surrounding tissues after fertilization, showing bicellular embryo. $\times 50$. Fig. 11. Embryo of Fig. 10 more highly magnified. $\times 226$. Fig. 12. Older embryo with two adjacent endosperm cells. $\times 226$. Fig. 13. Ovule with degenerate embryo-sac, in which the contents have been reduced to a small granular mass at the micropylar end. $\times 150$. Fig. 14. Degenerate ovary in which the whole nucellus has become disorganized, leaving a cavity surrounded by its integument. Fig. 15. Ovule in which the hypertrophied tissue of the nucellus occupies part of the cavity of the embryo-sac, thereby causing the endosperm tissue to assume an unusual shape. $\times 16$. A. Antipodals. c. Cavity formed by degeneration of the nucellus. EC. Endosperm cell. En. Endosperm nuclei. End. Cellular endosperm. Es. Embryo-sac. Em. Embryo. o.w. Ovary wall. M. Micropyle. N. Nucellus.

frequently met with in fertile ovules, was for the tissues of the nucellus to proliferate and occupy a considerable part of the embryo-sac cavity. This sometimes occurred even when the latter had become enlarged, as shown in Fig. 15. The curious shape which is assumed by the endosperm when the nucellar tissue grows into the embryo-sac cavity is worthy of note. In certain mature ovules in which this had happened the endosperm had become so contorted that in some of the sections it looked as though two separate lots of

endosperm tissue were present; but there can be no doubt that this appearance was caused by enlargement of the nucellus in the manner just described. In some instances seeds containing well developed embryos but with little or no endosperm were observed, whilst in others the endosperm became disorganized at an early stage. In yet others well developed endosperm was present but no embryos. It is quite evident that some of these abnormalities can give rise to apparently viable seeds which are incapable of germination. This has been fully confirmed by experiment. That the break-down of the embryo-sac or nucellus is not caused by lack of viable pollen was shown by the fact that long pollen tubes were frequently present even in those ovaries which had become most profoundly disorganized. In this connexion it may be mentioned that Sir Arthur Hill drew attention to the fact that, when working about thirty years ago with races of *R. Ficaria* which bore axillary tubercles, he found the percentage of apparently viable seed could be appreciably increased by cutting off the flowers above the highest tubercle and growing them in water. This has been fully confirmed in experiments carried out in collaboration with Marsden-Jones, but the number of seeds in which embryos are formed does not appear to be more than slightly increased by the treatment. Many of the seeds produced in this way will, therefore, fall within the apparently viable category which is incapable of germination. Kindler and Loschnigg (*loc. cit.*) also experimented with flowers which were cut off above the level of the tubercles and grown in water or culture solutions, and likewise report that the treatment increased the proportion of seed which appeared to be viable, but apparently no germination tests were made. Loschnigg mentions, however, that ovaries of some of the flowers treated in this way first became swollen but subsequently collapsed.

Two lots of flowers were collected in 1935 at random from plants with and without aerial tubercles respectively, and, after removal of the anthers, were grown in water in the breeding house at Potterne from which insects were excluded. After being artificially pollinated, fixations were made at intervals and the material examined microscopically at Kew. No noteworthy differences between the plants with and without aerial tubercles respectively were observed except that the presence of tubercles was constantly associated with a higher proportion of abnormal embryo-sacs. The abnormalities were of precisely the same types as those met with in the wild material already described.

In 1936 further pollination experiments, which with the one exception of series 6 (see p. 100) were on flowers still attached to the plants, were carried out by Marsden-Jones at Potterne, the material, as before, being fixed at intervals and sent to Kew for examination. The details of the treatments and the results of the microscopical examinations are summarized on pp. 100 and 101.

Ovaries from plants both with and without aerial tubercles respectively were examined. The pollination was controlled. Some of the plants were protected, the anthers being left intact so that self-pollination or apomixis were the only

possible preliminaries to the formation of embryos. Others were deliberately cross pollinated. Then the flowers in series 7 were protected after the anthers had been removed so as to ensure that any embryos which might be found in this material had arisen by apomixis. It must not be assumed that the proportion of ovaries which are shown in the table to be to a greater or less extent defective bears any exact relation to the ratio of complete to defective ovaries which would have been found in a random collection from the same plants. In actual practice one could with experience judge from their naked-eye appearance which of the ovaries were most likely to be completely disorganized. As there was no object in examining an indefinite number in which the nucellus and embryo-sac had broken down completely, a series of ovaries which were likely to show as wide a range as possible of stages of disorganization were selected from each plant; but with the older material special attention was paid to the swollen achenes. Examination of the table shows that the various types of abnormality in the ovaries are precisely the same as those already described, and, as before, they are most pronounced in plants provided with aerial tubercles. There are, however, a few interesting points to which attention may be drawn. For instance, the rate of development of the pollen tubes seems to be very variable, even when pollen from a single source is used to pollinate different plants. This is shown especially in series 2 and 3, where plants $\times 13.6$ and $\times 13.9$ respectively were pollinated by S.P. 87. Here the pollen tubes grew very much more rapidly in series 3 than in 2. There was no great difference in the proportion of swollen achenes in these two series at the time of fixation. It is also important to note that the central endosperm nucleus almost invariably gave rise to a layer of endosperm nuclei situated at the periphery of the embryo-sac at a time when the pollen tubes were still short, and before fertilization or triple nuclear fusion could possibly have occurred. Long pollen tubes which had developed very rapidly were, on the other hand, frequently to be seen in ovaries in which the nucellus had become decomposed. All of these facts tend to suggest that the development of embryos and endosperm is not necessarily preceded by normal fertilization, or, in other words, that the embryos sometimes arise apomictically from unfertilized eggs. Final proof that this is so was obtained both in the 1935 experiment at Potterne as well as with the plants in series 7 in 1936.

APOMIXIS

In the 1935 experiment the anthers were removed from the flowers in two lots of protected plants without aerial tubercles, which were not subsequently pollinated. On these a mixture of swollen and unswollen achenes was produced in the same way as with the pollinated material. No attention was paid to the early stages of development in this material, but ten of the swollen achenes were examined microscopically. In five of these there were small unhealthy embryos, and in two of them large well developed ones. From three of the largest achenes embryos were absent. Well developed endosperm was

Series Number.	Ref. No. of Plant.	Aerial tubercles present (P), absent (A).	Source of Pollen.	Fixation Date.	No. of ovaries examined.	No. of ovaries complete.	No. with egg apparatus reduced.
1	× 20·1	A	Protected but not deanthered. Self- pollinated or apo- mictic.	9/4/36 16/4/36 16/4/36 23/4/36 30/4/36 7/5/36 14/5/36 25/5/36 4/6/36	19 14 12 8 10 15 3 6 —	10 9 7 3 0 0 1 1 1	N N N N N N N N N
2	× 13·6	A	Cross-pollinated by plant S.P. 87	14/4/36 21/4/36 28/4/36 4/5/36 12/5/36 25/5/36	11 15 5 7 6 3	8 9 1 2 3 1	3
3	× 13·9	A	Cross-pollinated by plant S.P. 87	17/4/36 24/4/36 1/5/36 8/5/36 15/5/36 22/5/36	11 10 4 5 6 2	11 7 4 2 3 2	0 0 0 0 0 0
4	S.P. 58·2	P	Protected but not deanthered. Self- pollinated or apo- mictic	14/4/36 28/4/36 12/5/36 20/5/36	7 7 4 5	4 4 1 1	2
5	S.P. 59·4	P	Cross-pollinated by plant S.P. 87	18/4/36 2/5/36 9/5/36 17/5/36 23/5/36	9 7 5 4 3	6 4 2 2 2	1 0 0 0 0 0
6	× A ₃	A	Cross-pollinated plant S.P. 87. Cut flowers in water	22/4/36 6/5/36 13/5/36	8 6 7	7 3 2	0 0 0
7	× 36·1	A	Deanthered and protected. Apo- mixis alone possible	25/4/36 2/5/36 9/5/36 17/5/36 23/5/36	10 9 5 4 5	9 8 5 2 2	1 0 0 0 0
8	× 30·5	A	Open pollination	29/4/36 6/5/36 20/5/36 27/5/36	11 4 3 8	4 3 — 3	0 0 0 0

No. empty.	No. with break-down of nucellus.	No. with embryos.	Endosperm.	No. immature.	Pollen tubes.	Remarks.
3	0	0	Central nucleus	2	None	
0	0	0	" "	3	Short	
0	0	0	" "	3	"	2 complete embryo-sacs enlarged.
1	0	0	" "	2	"	
0	0	0	" "	0	"	
4	0	0	Peripheral in 1.	0	"	
14	0	0	Peripheral in 1.	0	"	
1	1	1	Peripheral	0	—	1 with peripheral endosperm col- [lapsed.
1	0	3	Solid"	0	Short	Endosperm but no embryo in 1.
		1		0	"	
0	0	0	Central nucleus	0	Short	Incomplete ovaries swollen.
2	1	1	Peripheral in 1	4	"	Egg connected to synergid by [cytoplasm in 2.
3	1	1	" "	0	—	
4	2	2	" "	0	Short	Nucellar tissue bulging into 1 e-sac.
1	3	3	Solid in 3	0	"	
2	1	1	" "	0	Long	
0	0	0	Peripheral in 2	0	Long	Egg connected to synergid by [cytoplasm in 1.
0	0	2	" "	2	"	
0	0	4	" "	0	"	
0	3	2	Contracted in 2	0	"	
0	3	3	Solid in 3	0	"	
0	0	2	" "	0	"	
1	0	0	Central nucleus	0	Long	No endosperm nucleus in reduced [e-sac.
0	3	1	Peripheral in 2	0	"	
0	3	1	" "	0	"	
0	4	1	" "	0	—	1 ovary collapsed after enlargement.
2	0	0	—	0	Short	Break-down of 1 e-sac after com- [pletion.
0	3	4	Peripheral in 4	0	—	
0	3	2	Partly disorganized	0	Long	Embryos in disorganized endo- sperm.
0	2	2	Peripheral	0	—	Achenes swollen but nucellus dis- organized.
1	0	2	Disorganized	0	—	Embryos in disorganized endo- sperm.
0	0	0	Peripheral in 1	1	Short	
0	3	3	" "	0	Long	
1	4	2	Disorganized	0	—	Embryos disorganized after de- velopment.
0	0	0	Central nucleus	0	—	Eggs connected to synergids by cytoplasm.
0	0	0	Peripheral in 1	0	—	2 ovaries somewhat swollen.
0	0	2	" "	0	—	Eggs connected to synergids by [cytoplasm.
0	2	2	Solid in 2	0	—	
0	3	2	" "	0	—	
0	0	0	Peripheral in 3	5	Short	
0	1	3	" "	0	"	
0	5	3	Solid"	0	"	
0	5	3	" in 1	0	"	2 embryos becoming disorganized.

present in all of them. This not only confirms the suggestion which has already been made that endosperm formation can occur without fertilization, but also affords definite proof that it is possible for embryos to arise apomictically, although in this instance the majority of them seemed to be somewhat unhealthy and liable to become disorganized.

In series 7 of the 1936 experiments much more convincing evidence of apomixis was obtained. Notes made at the time when the older material in this series was fixed indicate that a fair proportion of achenes were enlarged. Subsequent microscopical examination showed that all of the swollen achenes in this series of which sections were cut contained perfectly normal and healthy looking embryos. Hence it is reasonable to assume that a high proportion of apomictic embryos was produced. The existence of apomictic embryos is by no means unknown in the Ranunculaceae. Overton (1902), for instance, demonstrated that in *Thalictrum purpurascens* apomixis is a regular and normal occurrence. Marsden-Jones and Turrill (1935) have also shown that apomixis sometimes occurs in *Ranunculus acris*. In the present investigation no germination tests were made with seeds in which the embryos were known to have arisen apomictically.

THE FOLIACEOUS TYPE OF *R. FICARIA*

Ovaries from the so-called foliaceous type of *R. Ficaria* were also examined in 1936. Although the embryo-sacs of these appeared to develop in most instances, they were found to contain a varying number of large abnormal nuclei of which only the antipodals were generally recognizable. In others the embryo-sacs probably failed to develop altogether, but whatever may have happened in the early stages the nucellar tissues invariably collapsed sooner or later, disorganization starting in the region of the embryo-sac and spreading outwards. The entire nucellus frequently collapses leaving a cavity surrounded by tissues of the integument. The reduction of the gynoecium in the so-called foliaceous type does not, therefore, differ essentially from that found in other forms of *R. Ficaria*.

[In the present paper only those forms of degeneration which are met with in ovaries which appear superficially to be quite normal when young have been described. Examples of incomplete floral development, which are especially common in plants with aerial tubercles have been described previously. (Metcalfe 1938).]

SUMMARY

Lack of viable seed in *Ranunculus Ficaria*, which is especially characteristic of forms which bear tubercles in the axils of the caudine leaves, is chiefly due to various forms of degeneration in the embryo-sac or nucellus which are fully described. The fact that ovules frequently become disorganized, even when active pollen tubes are present, shows that degeneration is not due to lack of viable pollen. The process of fertilization in normal embryo-sacs is

described. Fertilization is not a necessary preliminary to the formation of endosperm, and definite proof was obtained that embryos sometimes arise from unfertilized eggs. Seed which is apparently viable is not always capable of germination for reasons which are fully described. Forms of *R. Ficaria* with axillary tubercles appear to be almost absent from certain localities in Devonshire and Somerset, although there are districts in other parts of the country where they are dominant. In other places forms with or without aerial tubercles appear to exist side by side in about equal quantities. In parts of central Europe, records show that the species almost invariably bears axillary tubercles.

I am indebted to all those who have assisted in various ways with this investigation. Thanks are due especially to Sir Arthur W. Hill for his interest and advice in the preparation of the manuscript. Also to Mr. E. M. Marsden-Jones who carried out the pollination experiments, fixed material, and supplied living plants from the Potterne Biological Station. Dr. W. B. Turrill's suggestions and criticisms have also been most helpful. I am also grateful to Mr. G. Atkinson for advice and assistance in preparing the illustrations.

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The Morphology and Anatomy of *Pellia epiphylla* considered in Relation to the Mechanism of Absorption and Conduction of Water

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With four Figures in the Text

PELLIA EPIPHYLLA is a very common liverwort growing in a variety of situations varying from hedges to damp ditches and banks of streams, and it may be even submerged. The thalli are often entirely prostrate but, where the plants are very crowded, the younger portions become sub-erect, and this tendency allows for a certain amount of overlapping of the plants. The method by which water reaches the tips of these inclined apices was first investigated.

Plants, having previously been washed and freed from surplus moisture by means of blotting-paper, were placed on filter papers on Petri dishes so that they were inclined at angles of 35° to 40° to the horizontal, their tips resting against the edges of the dishes, with only their distal ends in contact with the filter paper. These filter papers were saturated with 0.5 per cent. solutions of various 'vital' stains, such as vital red, methylene blue, neutral red, trypan red, pyrrol blue, and diamin black respectively, the last-named stain having been dissolved in a 1.0 per cent. solution of sodium chloride. Sufficient stain was used to ensure the presence of a film of it over the filter paper. Each Petri dish was enclosed within a glass chamber to maintain a moist atmosphere (Fig. 1).

At the end of an hour the plants were examined, and it was noticed that in every case the stain had reached the tip of the thallus, and had stained the rhizoids. The portions of the plant not provided with rhizoids, e.g. the 'lamina', were quite free from stain. The rhizoids were, therefore, obviously effective in conducting capillary films of liquid externally from the filter paper to the apices, as Bowen (1935) had found in the case of *Fimbriaria bleumeana*.

Further experiments showed the conduction of liquid in this rhizoidal region to be exceedingly rapid. The time taken to travel from base to tip was determined by means of a stop watch, and typical results obtained are shown in Table I.

It is obvious from the data that *Pellia epiphylla* has a very rapid conduction, and that this will be very effective in providing the inclined tips of the thallus with water from the soil.

The effect of preventing this external conduction was tested by carefully removing the greater number of rhizoids and covering the whole thallus with a thin layer of vaseline. A fresh surface was then exposed at the base by a clean cut with a scissors, and plants so treated were placed as before, in

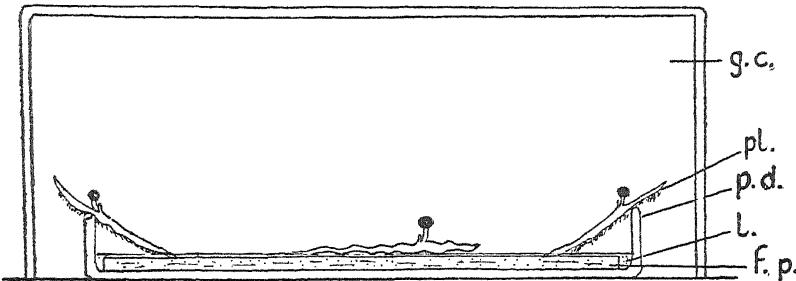


FIG. 1. Diagram of apparatus used. *g c.*, glass chamber; *pl.*, plant; *P d.*, Petri dish; *f p.*, filter paper; *l.*, liquid.

inclined positions in Petri dishes containing a 0·1 per cent. solution of potassium nitrate. After a time sections were cut at various points on the thallus and were mounted in diphenylamine dissolved in concentrated sulphuric acid. They were then immediately examined for the characteristic blue coloration denoting the presence of the potassium nitrate in the thallus tissue.

TABLE I

Plant number.	Length of plant (cm.)	Time taken by stain to reach tip of plant.
1 (vital red) . . .	2·6	1 min. 4 sec.
2 " " . .	2·9	1 min. 7 "
3 " " . .	2·4	1 min. 20 "
4 (neutral red) . .	2·0	— 58 "
5 " " . .	2·6	1 min. 3 "
6 " " . .	2·7	1 min. 11 "
7 (methylene blue) . .	2·4	— 57 "
8 " " . .	3·0	1 min. 5 "
9 " " . .	2·7	— 47 "
10 (Pyrrol blue) " .	3·0	— 36 "
11 " " . .	3·2	1 min. 10 "
12 " " . .	2·9	— 58 "

Other plants similarly prepared were placed in dishes containing a 0·1 per cent. solution of ferric chloride, and the sections mounted in ammonium sulphide solution. A black precipitate of sulphide of iron occurred wherever the ferric chloride solution was present in the tissues. Typical results obtained by these methods are shown below.

It was evident that the rate of internal conduction, as revealed by the above methods, was very slow, far slower than the rate of external conduction, and it would appear unlikely that it would suffice to supply more than a fraction of the needs of the plant.

An attempt was then made to determine the region of entry obvious, Thalli, in an inclined position, were again placed in dishes containing 5% cent. solutions of the stains mentioned above. At the end of one hour the plants were washed and transverse sections were cut and examined. These

TABLE II

Plant number.	Length of plant (cm.)	Time.	Height (cm.) to which potassium nitrate had risen internally
1	1·9	6 hrs.	0·3
2	2·0	”	0·2
3	3·4	24 hrs.	0·4
4	1·8	”	0·3
5	2·0	3 days	0·6
6	2·2	”	0·7

TABLE III

Plant number.	Length of plant (cm.)	Time.	Height (cm.) to which ferric chloride had risen internally
1	2·1	6 hrs.	0·3
2	1·6	”	0·3
3	2·0	24 hrs.	0·5
4	2·3	”	0·6
5	1·8	3 days.	0·9
6	2·0	”	0·8

showed that the liquid had passed up between the rhizoids, had entered into the rhizoids to a certain extent, and had passed into the lower layers of cells on the ventral surface of the 'midrib'. No coloration was, however, to be seen in the upper part of the 'midrib' or in the wings, when the plants had been in the dishes for one hour only. After longer periods all the tissues became coloured with the respective dyes, which spread first in the upper part of the 'midrib', aided, no doubt, by the numerous mucilage cells present, and thence into the 'lamina'. When thalli were placed prostrate on the saturated filter papers the whole under surface in contact with the films of stain became stained much more rapidly. The whole under surface is, therefore, capable of absorption.

The preceding experiments were now repeated using fertile thalli, in which it was noticed that the margin of the thallus becomes slightly 'frilled' and markedly incurled in the region of the antheridia, but flattens again towards the archegonial tip (Fig. 2).

When such thalli were inclined at angles of 35° to 40° the stains not only travelled along the under surface of the 'midrib', but also spread rapidly in thin films over the upper surface, filling the antheridial cavities and bathing the antheridia, but never reaching the involucrum or the archegonia. The spreading of the liquid over the upper surface was obviously facilitated by the incurving of the margin in the region of the antheridia, these margins and the projecting margins of the antheridial cavities presenting considerable surface for the retention of films of water. Sections of such plants showed that the antheridial

central cells of the seta, the elaterophore, the spore walls, elaters, and a certain amount of the mucilagenous matrix between the developing spores and the elaters, became coloured, before the tissues of the gametophyte in the neighbourhood of the foot showed any appreciable staining.

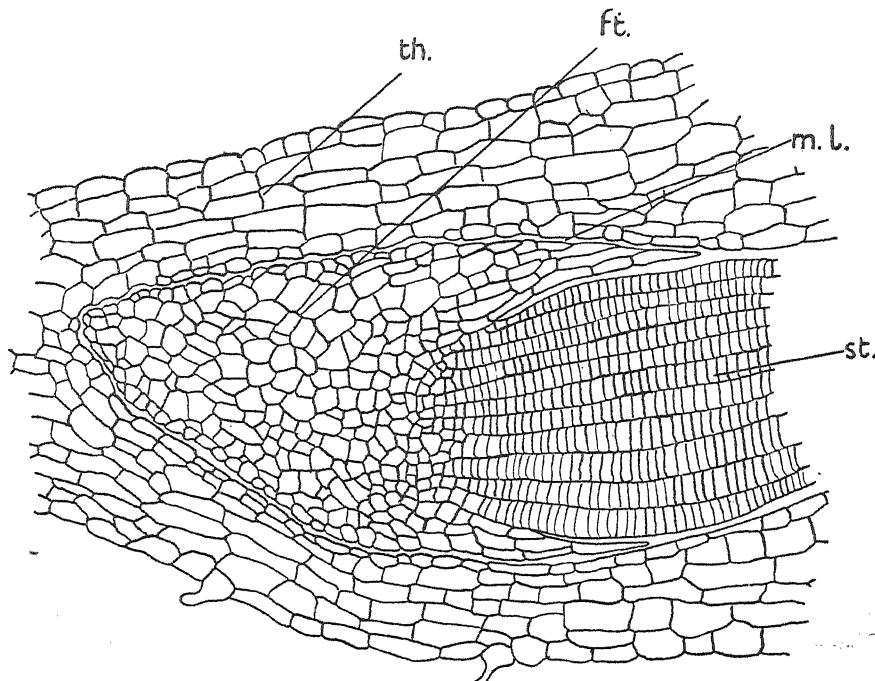


FIG. 4. Longitudinal section of *Pellia epiphylla* showing the presence of a thin mucilage layer separating the foot from the gametophyte. *th.*, thallus; *ft.*, foot; *m l.*, mucilage layer; *st.*, seta. $\times 100$.

It is therefore clear that the maturing sporophyte of *Pellia epiphylla* receives the greater part of its water supply from external sources and not from the gametophyte. How far these findings apply to other members of the Bryophyta is under investigation.

SUMMARY

1. In *Pellia epiphylla* water travels in the form of capillary films between the rhizoids over the under surface, being partly absorbed there. The remainder passes over the surface and is retained in the region of the antheridia by the incurved and wavy margins of the thallus.

2. The antheridia, oospheres, segmenting oospores, and sporogonia are largely supplied with water from the exterior. This water is retained in the narrow crevices between antheridial cavity and antheridium, between the flaps of the involucre, and between these and the long necks of the archegonia. Travelling down the necks of the archegonia, it reaches the oospheres, and is

later available for the developing sporophyte in the form of films retained between the foot of the segmenting embryo and the calyptra.

3. In the sporophyte, this water, absorbed by the foot largely from external sources, travels up the seta, into the elaterophore, out to the elaters, and ultimately to the spores.

4. The sporophyte, therefore, is much less dependent on the gametophyte for supplies of water and raw materials than might be expected.

The writer wishes to take this opportunity of thanking Professor F. A. Mockeridge for suggesting this work and for her valuable advice and criticism during its progress.

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The Inheritance of Growth Rate in *Neurospora crassa* with Special Reference to Hybrid Vigour and Cytoplasmic Inheritance

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With seven Figures in the Text

1. INTRODUCTION

A FUNGUS was selected as being more suitable than a flowering plant for purposes of determining the inheritance of certain physiological characters. The reasons for this were twofold. First, the ease with which growth rates can be determined in a fungus under diverse controlled conditions, and second, the great simplification in interpreting results in the haploid as compared with the diploid generation owing to the absence of dominance. *Neurospora crassa* was chosen as the most promising type available owing to the large amount of work (Lindegren, 1933, 1936) which had already been carried out with it on the inheritance of morphological characters.

The following account deals with the inheritance of growth rates in eight different crosses. In three of these details of inheritance are recorded and in one cross the problem of hybrid vigour is considered in the light of observations on optimal growth conditions.

2. EXPERIMENTAL PROCEDURE

The strains of *Neurospora crassa* were those used by Dr. C. C. Lindegren, to whom I am greatly indebted for the original cultures. Eight strains were received from him, namely, *pale-*, *crisp-*, *dirty-*, *gap+*, *soft+*, *dirty-gap+*, *gap-pale+* and the cross *gap-pale+ × normal-*. Each strain was subcultured from a single hyphal tip. Stock cultures were kept on test-tube slopes and great care was taken to prevent admixture of strains during subsequent subculturing. Throughout the work malt-agar (2 per cent. malt, 2 per cent. agar) was used as the culture medium and was found eminently satisfactory both from the point of view of growth-rate determinations and the differentiation of the morphological characters of the various strains.

In making a cross subcultures of the two strains were placed about 1 cm. apart on a test-tube slope and incubated at 30° C. for some three to four weeks, by which time the perithecia produced contained mature ascospores.

To obtain ascospore germination the following procedure was adopted which is in general similar to that advocated by Lindegren (1932). Several perithecia were taken and crushed between two microscope slides previously sterilized. The loose ascospores and broken-up perithecial walls were then washed into a test-tube with water and well shaken. This suspension was poured over several dishes containing malt-agar and the surplus drained off. The dishes were incubated for four hours at 30° to allow conidia present to germinate: they were then placed in an incubator at 59° for one hour and subsequently overnight at 30° C., when colonies arising from germinated ascospores were subcultured each on to a test-tube slope. Tests showed that this method was completely satisfactory in killing conidia, so the resulting colonies were derived entirely from germinated ascospores. The latter point was verified by microscopic examination of a large number of colonies which in every case were found to have originated from ascospores. Between 50 and 70 ascospore colonies were subcultured from each cross.

Test-tube slopes were used when comparing the various segregating types morphologically, as the different characteristics of the strains were found to show up much more clearly in these than in Petri-dish cultures. Determinations of growth rates were made in Petri dishes and were carried out generally in triplicate, though in certain cases, for example in the cross 26×88, five replicates of each strain were grown. The inocula were placed at the sides of the dishes, which were then incubated at 36° C. overnight. The distance from the edge of the dish against the inoculum to the growing edge of the colony was determined the following morning, using the apparatus previously designed for a similar purpose (Dickson, 1935), and another measurement was made six hours later. This method was necessary owing to the extremely rapid rate of spread of some of the strains, a rate of advance of as much as 4–5 cm. in eighteen hours having been recorded. The mean increase in radius, in six hours, of the various replicates of each segregant forms the basis on which the curves of Figs. 1, 2, 3, and 4 have been constructed. Tests showed that the standard errors of the means obtained by this method were about 3 per cent. of the latter. In the case of Fig. 5, where the strains are all comparatively slow-growing, the inocula were placed in the centres of the dishes and the diameters measured after two days' growth at the various temperatures.

Fig. 1 shows the frequency distributions of the growth rates of the segregants of each of the eight crosses. The two circles above each frequency curve represent the growth rates of the parents of the cross. In Figs. 2, 3, and 4 the segregants have been sorted into distinct morphological types and frequency distributions of the types are recorded each on its own ordinate. That similar figures have not been given for the other five crosses is due to the fact that the number of F_1 types in each cross lay between 8 and 16 (due probably to linkage combined with the comparatively small number of segregants examined) and a satisfactory factorial scheme could not therefore be

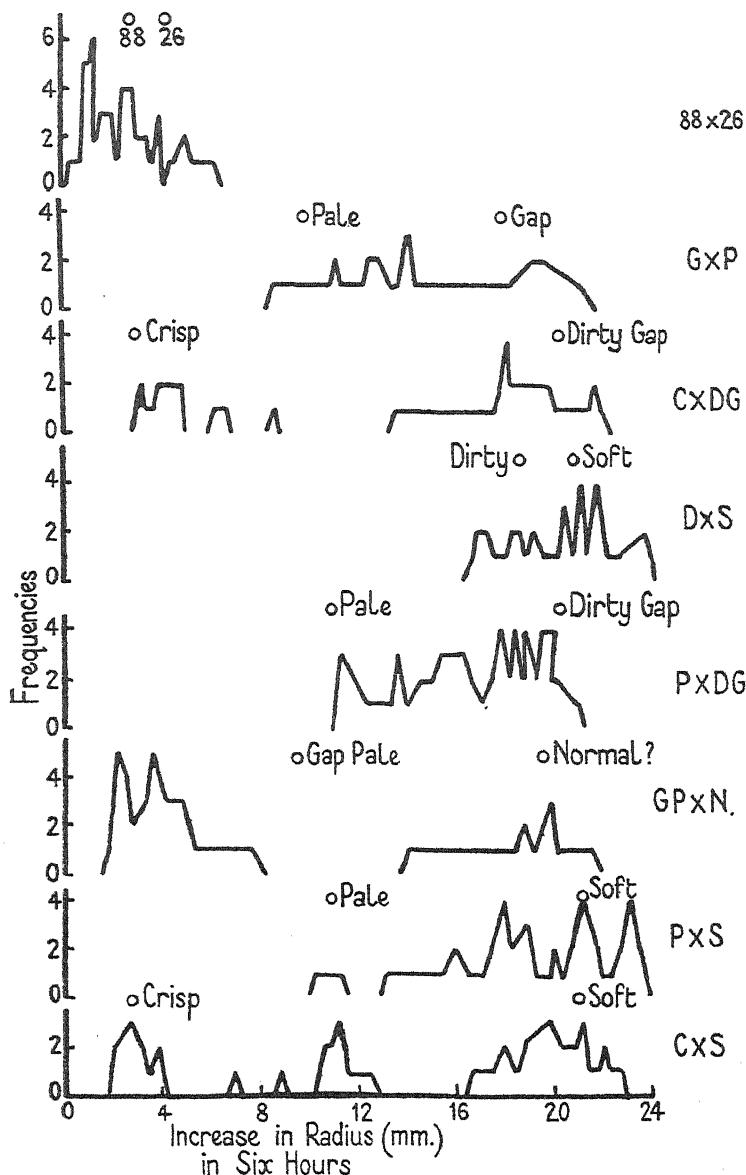


FIG. 1. For explanation see text.

obtained. Fig. 5 shows the growth rates in two days of eight F_1 strains and their two parents at various temperatures.

3. RESULTS

Stability of the fungus.

Lindegren (1936) has shown that mutant types are occasionally found in pure culture, but during the two years which have elapsed since the original strains were received by the author these have remained true to type. No mutant sectors have been observed in any of the hundreds of cultures which have been grown during that time. It is therefore of interest to note that in six of the seven crosses which have been made with the original cultures more F_1 types have been found than were to have been expected from the genotypes of the parents as scored by Lindegren. Table I shows the numbers of morphological types actually obtained and the numbers which the genotypes of the parents would lead one to expect.

TABLE I

Cross	$s \times d$	$dg \times p$	$c \times dg$	$s \times p$	$p \times g$	$c \times s$	$gp \times n$	26×88
F_1 types expected :	4	8	8	4	4	4	8	?
F_1 types found . . .	13	10	12	8	12	4	15	8

It is thought that these discrepancies may be due to different methods of culturing the strains. To judge from the photographs reproduced (Lindegren, 1936, Plate XIV), Lindegren's strains were compared when growing in un-sloped test-tube cultures, whereas in the present case slopes have been invariably used and many of the characteristics distinguishing the different strains are probably only to be seen on a sloped medium. For example, marked colour bands appear at the bottom ends of the slopes in some strains but not in others, and in some, masses of spores are produced at the thin top end of the slope, whereas in other strains they are evenly dispersed over the surface. It is suggested that this difference in technique may account for the greater number of types recorded here.

General relationship of parental and F_1 growth rates.

The frequency distributions of the segregants of each of the various crosses together with the mean rates of the parents are shown in Fig. 1. It will be seen that as regards parental growth rates all types of crosses have been made, thus $c \times s$ and $c \times dg$ represent crosses between fast- and slow-growing parents. $p \times s$, $gp \times n$, and $p \times dg$ have each an intermediate and a fast parent. In the cross $p \times g$ both parents have a medium growth rate and $d \times s$ and 26×88 are crosses between two fast and two slow parents respectively. With the exception of the cross $gp \times n$ the majority of the strains from each cross have growth rates between those of their parents; in this cross, however, more than half the strains have growth rates slower than that of the slower parent gp . In most cases there are larger numbers of strains with growth rates near to those

of their parents than there are at other points. The crosses $gp \times n$ and $p \times s$ are, however, exceptions to this, as in the former case there are no strains with growth rates equal to that of gp , and in the latter only very few with

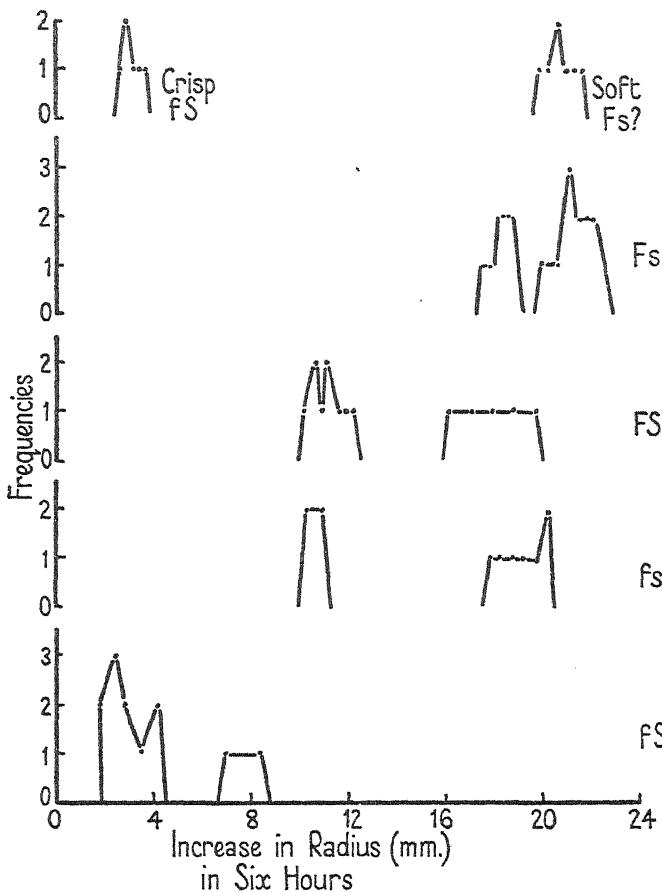


FIG. 2. For explanation see text.

rates similar to p . These exceptions will be dealt with later when the question of cytoplasmic inheritance is discussed. Few segregants have growth rates significantly greater than that of the faster of their respective parents. Where this is so, and excepting certain strains in the cross 26×88 , the faster strains have a much thinner mycelial development than their faster parent.

Segregants of the crosses $c \times s$, $p \times s$, and 26×88 .

The results of the cross $crisp \times soft$ are shown in Fig. 2. (As the number of types of segregants exceeded the number expected, considerable difficulty

was experienced in determining which character referred to *crisp*, which to *pale*, &c., consequently the use of these names has been discontinued and new ones have been applied to the different F_1 types.) Two pairs of factors, *fluffy* (*F*), *non-fluffy* (*f*), and *sporiferous* (*S*) *non-sporiferous* (*s*), were found to be present giving the four types *FS*, *Fs*, *fS*, and *fs*. These groups were probably not morphologically homogeneous, but such differences as were observed between the strains of each group were small and indefinite, and it was not found possible to subdivide the groups further. The figure shows that there are two distinct frequency distributions for each morphological type indicating the presence of a factor affecting the growth rates. The strains *fS* were similar to the parent *crisp*, but the other parent *soft* was more fluffy than the *Fs* type segregants.

Fig. 3 shows the results of the cross *pale* \times *soft*. Three pairs of factors *Ff*, *Ss* (as above) and *Bb* are represented in all the eight possible combinations. The factor *B* is indicated by the presence of a well-defined orange band consisting of a thick weft of hyphae with spores, at the base of the test-tube slope, *b* indicates the absence of such a band. As in the cross *crisp* \times *soft*, each morphological type is represented by two frequency (growth rate) distributions indicating the presence of a growth-rate factor. Morphologically the groups were very homogeneous, and especially was there no observable difference between the two growth-rate types of each group. The parent *soft* was similar to the segregants *FBs*, but *pale* was markedly unlike *fbS* or any of the other segregants.

The strains 26 and 88 were chosen as slow-growing segregants from the crosses *gp* \times *n* and *c* \times *dg* respectively. The result of the cross between these slow-growing parents is shown in Fig. 4. Eight morphological types were obtained, the members of each being in all respects exactly alike. These types represent all combinations of the three pairs of factors *sporiferous* (*S*) *non-sporiferous* (*s*), the presence (*E*) or absence (*e*) of a thick weft of spore-bearing hyphae at the top edge of the slope, and the presence of 'rings' (*A*) and their absence (*a*). Rings were formed in some strains following the removal of the cultures from the incubator for several hours, during which time they were examined and left on the laboratory bench. It was subsequently found that rings were formed in those strains in which the optimum temperature for growth rate was low, i.e. about 30° , and which grew very slowly at the temperature at which they had been incubated, namely 36° C. Strains with optima at 36° (*asE* and *aSe*) and those with optima at 30° or just above, but which grew almost as rapidly at 36° (*aSE* and *ase*), did not produce rings. *Non-sporiferous* strains were not entirely free from spores, those present were distributed evenly over the surface of the colony in the absence of *E*, i.e. in *se* strains, but were concentrated at the top edge of the slope in *sE* types. No growth-rate factor such as was found in the two preceding crosses was present. There appears to be no correlation between growth rate and the presence or absence of any of the three morphological factors or of any

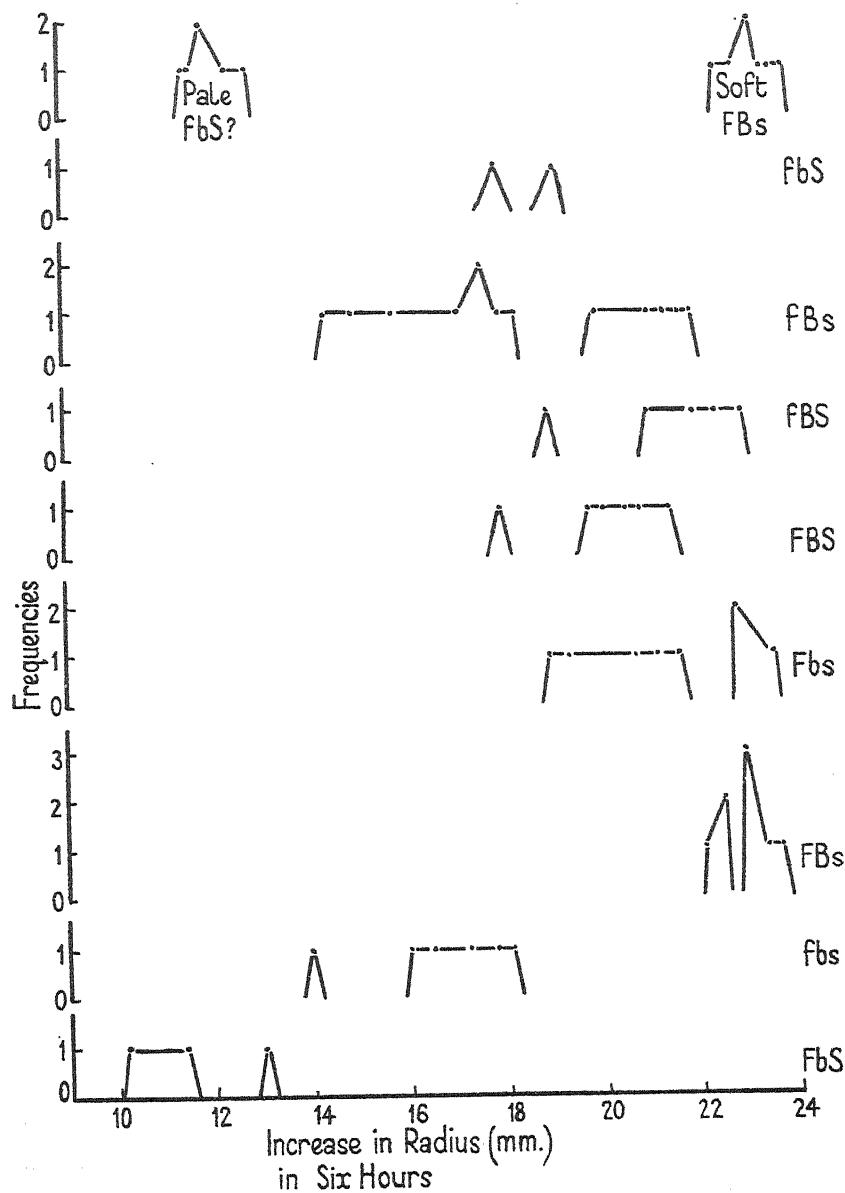


FIG. 3. For explanation see text.

combination of two of them. Strain 26 was similar to type *aSE*, but strain 88 was unlike any of the segregants not excluding *Ase*.

Segregation of types with different temperature optima.

The observation that rings were formed in some strains of the cross 26 × 88, as previously described, raised the question as to whether they were caused by the short exposure to light or to the lower temperature. It was found on growing two strains of each of the eight different morphological types at various temperatures that those strains which produced rings grew more slowly at 36° C than the others. It seemed probable therefore that the rings were due to the fall in temperature on removing the cultures from the incubator. Fig. 5 shows the variation of growth rate with temperature of one strain from each morphological group (the second strain from each group is not shown for clarity, but the results were similar to those recorded). The growth rates of the parents are shown at the top of the figure. Strain 88 grew so rapidly that in two days it had more than covered the Petri dish, hence the dotted line between 25° and 30° C. Growth measurements for this strain were consequently repeated and measurements made after twenty-four hours' growth are recorded.

The graphs show that of the two parental strains 88 has an optimal growth temperature at about 30° and grows very slowly at 36°, whereas strain 26 which has an optimal temperature at 30° to 33° grows almost as rapidly at 36°. The segregants fall into four types as regards the effect of temperature. First, strains *ASE* and *Ase* have an optimal temperature at 25° or below and grow progressively less rapidly at each higher temperature. Second, strains *ASe* and *AsE* have optima at 30° or just below and grow much less rapidly at 33° and 36° C. Third, strains *aSE* and *ase* have optima between 30° and 33° and grow almost as rapidly at 36°. Fourth, strains *asE* and *aSe* have optima at about 36° and are able to grow even at 40° C. It will be seen that whereas the parent 26 is identical in its growth reactions with type *aSE*, there is no type among the segregants at all comparable with the other parent, 88.

As only two strains from each morphological group have been examined it is not possible to draw any definite conclusions as to the number of factors which affect the growth rates of the strains at different temperatures or the manner in which such factors may take effect, i.e. in controlling optimal temperatures, the slopes of the curves between certain temperatures, &c. It can, however, be stated that certainly two and probably more factors are concerned and it is likely that they affect the growth reactions in different ways.

Hybrid vigour.

Of the original seven strains four (*s*, *d*, *gp*, and *dg*) had a common optimal growth temperature of about 36° and three (*g*, *p* and *c*) an optimum between

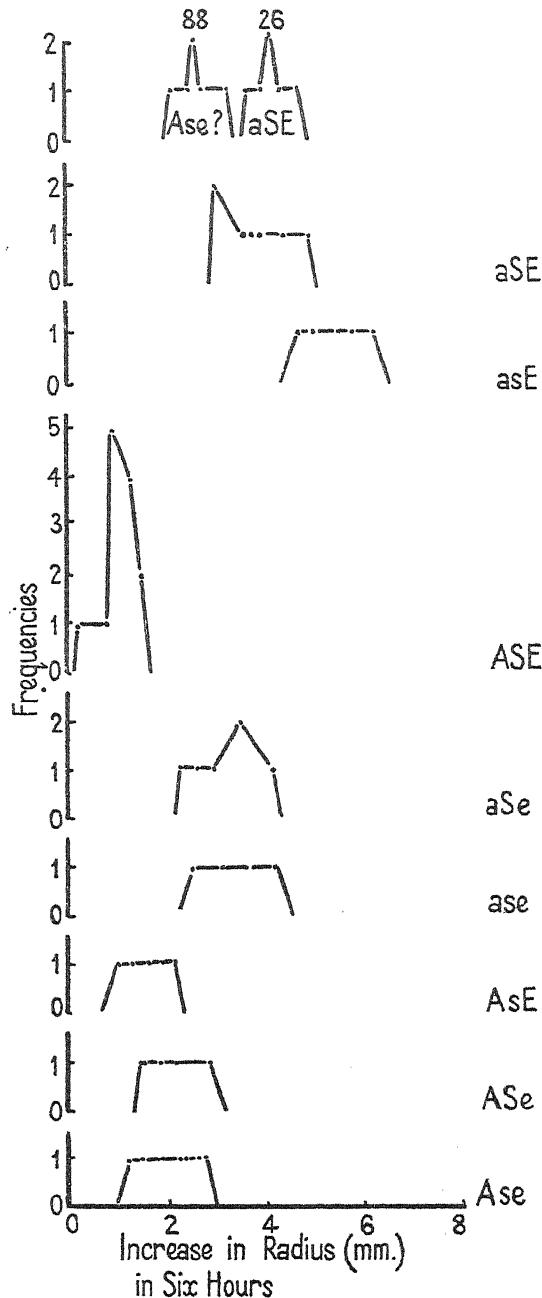


FIG. 4. For explanation see text.

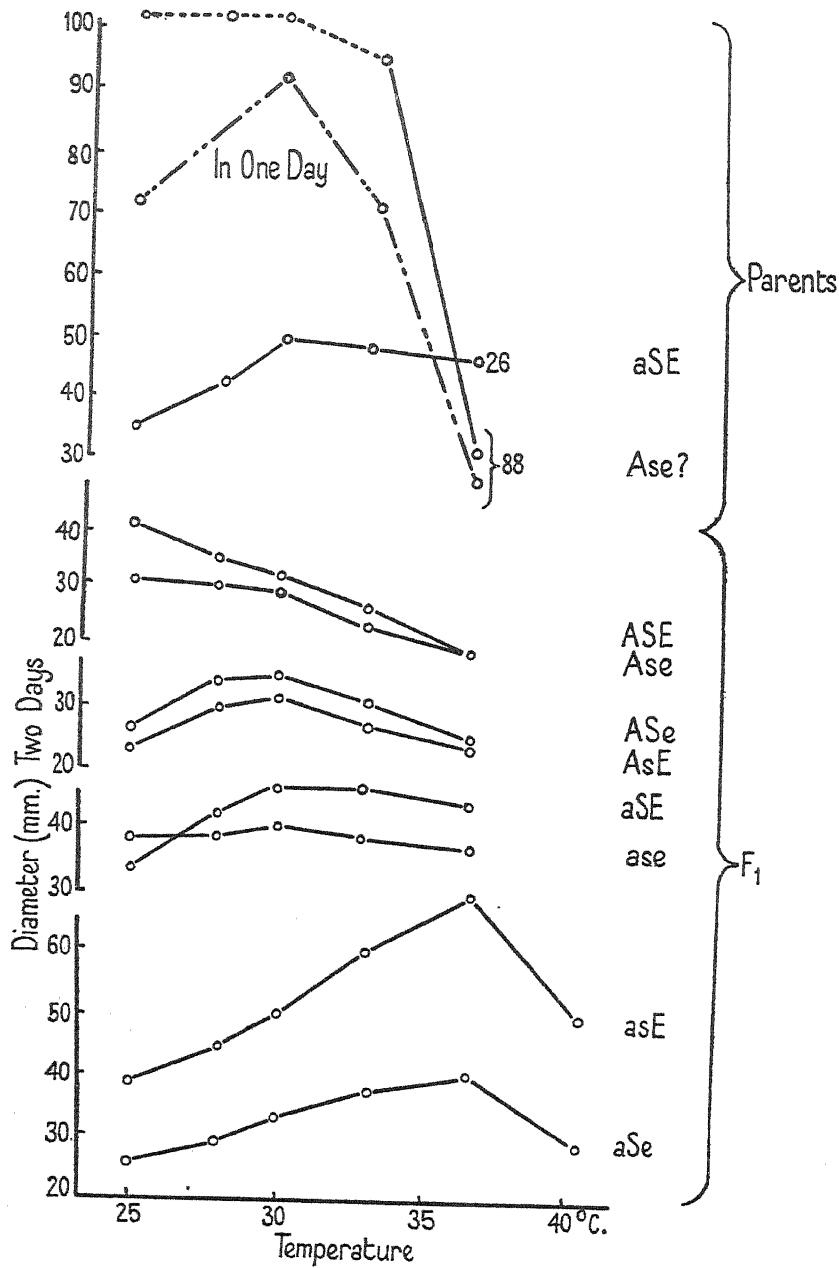


FIG. 5. For explanation see text.

30° and 36° C. It is for this reason that 36° was chosen as the temperature at which all growth measurements were made. As already pointed out, in a number of crosses segregants were obtained which had a greater rate of spread than either of their parents but this was accompanied by a thinner mycelial development. In the cross 26×88, however, type *asE* grows considerably faster than either parent at 36° and at the same time has a much more plentiful hyphal development. It would therefore appear to show marked hybrid vigour at this temperature, which at the same time was the optimal temperature of two, namely *gp* and *dg*, if not three (the optimum growth temperature of *normal* is not known as this strain was not received and has not since been definitely identified) of its four grandparents. While this is so it is nevertheless obvious that at the respective temperature-optima of the various types and of the two parents, and in fact at any temperature below 36°, no hybrid vigour is manifest. The position is still further complicated by a study of the results shown in Fig. 6, in which the growth rates of those segregants carrying the *A* factor and of the parent 88 are plotted against time. It is seen that while the four *A* bearing types have growth rates which remain practically constant for five days, the curve of strain 88 (continuous line) shows a rapidly decreasing growth rate. It is evident then that any question concerning the inheritance of physiological characters, in which is included the question of hybrid vigour, must be considered in the light of an extensive study of the growth of the strains concerned under various environments and not from determinations made under a single set of external conditions. In the present instance it is clear that on the evidence of the results shown in Fig. 4, an example of hybrid vigour would seem to have been established; this has been shown to be entirely illusory and merely due to the different growth responses of the strains examined.

Cytoplasmic inheritance.

As in the present instance the segregants are haploids, the numbers of the different types of segregant should be equal in the absence of linkage. Where linkage is present, as has been shown to be the case in the strains of *Neurospora* examined by Lindegren, the segregating types similar to the parents should have been in excess of other types. This, however, is not the case in most of the eight crosses here considered. Fig. 1 shows that in several cases there are no, e.g. *gp*×*n*, or relatively few, as in the cross *p*×*s*, segregants with growth rates comparable with one or other parent. Secondly, in the crosses *c*×*s*, 26×88, and *p*×*s* there are no segregants similar to the parents *s*, 88, and *p* respectively. Moreover, it has been found in the case of the first two crosses and probably also in the case of the cross *p*×*s* that perithecia were only formed on the *c*, 26, and *s* mycelia when the strains were paired. These facts all lead to the conclusion that cytoplasmic inheritance is almost certainly present, and that it plays a major part in determining not only the appearance but also the growth rates of segregating types. The strain on

which the perithecia are formed presumably acts as the maternal parent, and transmits cytoplasmic characteristics both morphological and physiological to the next generation. In further support of this contention it is noteworthy that in the three crosses $c \times s$, 88×26 , and $p \times s$, those types which according to the factorial scheme should be similar to the paternal parent (the types F_s ,

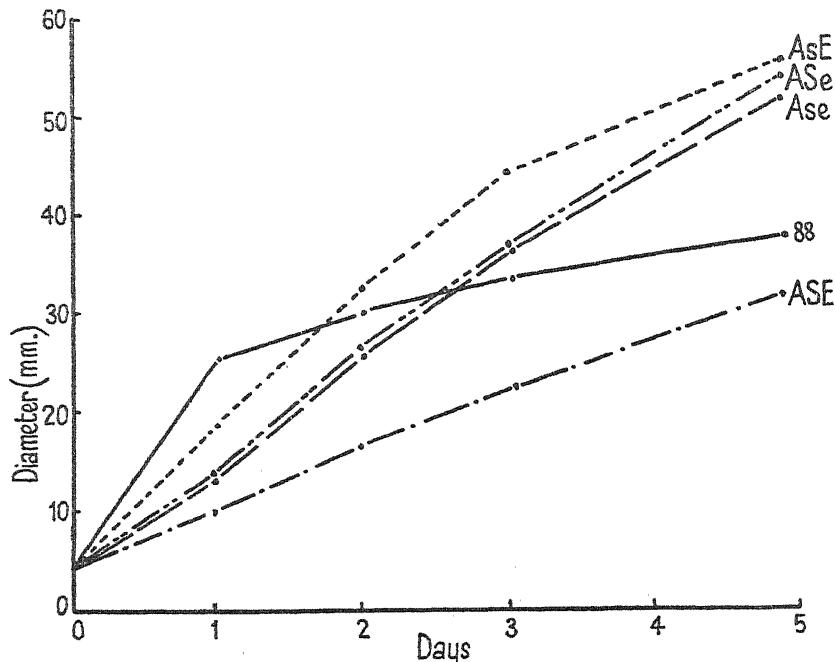


FIG. 6. For explanation see text.

Ase , and fbS respectively) are in effect identical with it from the point of view of the factors concerned, but otherwise differ from it in certain respects in which they more nearly resemble the maternal parent. As an example of this in the cross 26×88 the genotype of the paternal parent 88 is undoubtedly Ase , which is in agreement with what is required for the factorial scheme. The segregant type Ase , however, is readily distinguishable from 88 in that its mycelium is not so plentiful and its growth rate and temperature reactions are different. Both in appearance and in its physiological reactions, then, it tends to resemble the maternal parent, 26 . Generally it may be concluded that while the inheritance of certain characters is the same whichever parent is the maternal one, other characteristics, both morphological and perhaps to a major extent physiological, are determined or at least modified by the cytoplasm.

Analysis of a single segregant type.

It is not generally possible with the higher plants to compare statistically the growth rates of the individual members of a segregating group, so in view

of the complex results obtained on analysing certain data described above it was decided to determine more accurately than had been done the rates of spread of the individual members of a fast-growing group of five strains from the cross $s \times d$. The five strains selected were exactly similar morphologi-

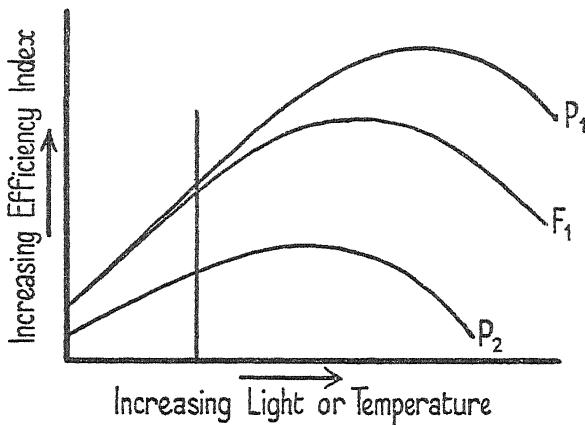


FIG. 7. For explanation see text.

cally. Ten subcultures were made from each and the rates of spread (radial) in six hours are shown in Table II.

TABLE II

Strain number.	Growth rate (mm.)	S.E.
4	25.9	± 0.37
8	24.7	± 0.15
2	23.8	± 0.40
5	23.1	± 0.17
17	23.0	± 0.15

The table shows that while some pairs of strains do not differ significantly in growth rate others do, and it is concluded from these results that the various morphological groups are probably not strictly homogeneous but are composed of strains which differ in certain minor characters.

4. DISCUSSION

In none of the three crosses studied can any of the morphological factors be shown to have a definite effect on the growth rate, in the sense that while the growth rates of the various morphological types are different, no one factor can be said either to cause an increase or a decrease in the rate of spread. It seems probable that this may be due to excessive interaction between the various factors, a situation to be expected owing to the extremely simple morphology of the fungal colony. On the other hand, there have been found in the crosses *pale* \times *soft* and *crisp* \times *soft* definite growth-rate factors which

give two distinct frequency distributions for the growth-rate measurements of each morphological type. These factors have no effect on the morphological characters.

Ashby (1930 and 1932) and Luckwill (1937) working with maize and *Lycopersicum* respectively have found that in no case did the 'efficiency index' of the products of a cross exceed that of the parent with the greater index. A somewhat analogous case has been established for the segregants of *Neurospora crassa* in that a greater growth rate combined with a similar or greater thickness of mycelium than the parent has not been found in any segregant of the eight crosses studied—the apparent exception to this statement, the cross 26×88, will be considered later. So far then as these experiments are concerned there would appear to be an upper limit to the rate at which the plant can increase in size. This limit must be determined either by the genes themselves or by the cytoplasm, and it has been shown in the present instance that the latter has a very marked effect not only on growth rate but also, to judge from the results of the cross 26×88 in which the parent 88 differs so widely from all the segregating types, on other physiological characteristics.

Each generation of plants is subjected during its development to two sets of environmental factors. There is firstly the effect of the maternal genotype which must be presumed to have some effect on the developing embryo, and in this effect should also perhaps be included that of the endosperm. Secondly, each plant is subjected to the very varied and complex effect of the external environment. That the latter factor is of major importance in all questions concerning growth inheritance is clearly shown by the results described in the present paper where an apparently straightforward case of hybrid vigour has been shown to be due largely to the response to external conditions of the strain concerned being totally different from that of either of its parents. In the case of Ashby's experiments on maize and those of Luckwill on tomato it would be of considerable interest to know the response of the efficiency indices of their parental and F_1 strains to temperature and light intensity changes. Both are warmth- and light-loving plants and were probably growing under conditions where these factors were far below the optimum. It might be that while at low light intensities and at low temperatures the growth index of the F_1 plants was similar to that of one of the parents (the faster growing one), under more optimal conditions it was intermediate between those of the parents. Such a possibility is illustrated by the theoretical curves shown in Fig. 7, where P_1 , P_2 , and F_1 represent respectively the efficiency curves of two parents and of the segregants derived from their union. Any measurements taken at light intensities or at temperatures below some point such as that shown by the vertical line in the figure would give results similar to those obtained by Ashby and Luckwill. Should this prove to be so it would explain the rather unexpected result of the relative growth rate—a function

influenced by so many factors—being inherited apparently as a simple dominant factor.

Returning to the question of cytoplasmic inheritance, Ashby has found a difference in hybrid vigour between reciprocal crosses in maize, and this he suggests may be due to the 'influence of the mother plant during maturation of the seed'. Such an influence might be due either to the effect of the maternal cytoplasm or the maternal genes, or to both acting together. In this case there is no evidence as to whether there is any difference between the cytoplasms of the parents so that it is impossible to draw conclusions concerning its possible effect. In the case of Luckwill's experiments, while a cytoplasmic difference might be expected between types so far removed as *Lycopersicum racemigerum* and *L. esculentum*, there is no account of a reciprocal cross having been made. So far as the author is aware no experiments have been performed whereby the effect of the cytoplasm alone on the various physiological measurements associated with growth have been made. It is suggested that such information might yield valuable light on these problems.

Cytoplasmic differences have in most cases been demonstrated between such high taxonomic units as families and genera. Several cases have, however, been recorded between species and subspecies (Sirks, 1931, 1932) and between tomato varieties by Schröder (1935). The latter examples are, however, not common and the present account of cytoplasmic differences in mutant types (the majority of which have originated during laboratory culture) of *N. crassa* is consequently of interest. As cytoplasmic inheritance here has been established entirely in the haplophase it is noteworthy that Goldschmidt (1938), in reviewing the influence of the cytoplasm on gene-controlled characters, states that it is 'a remarkable fact that the cytoplasmic influence is stronger upon haploid characters'.

In view of the absence of an efficiency index greater than that of the parents in the results recorded above, it is of interest to review certain results obtained some years ago during experiments on *Coprinus sphaerosporus* (Dickson, 1935). Three crosses are recorded, namely 10×2 , 15×16 , and 10×21 (in the latter case reciprocal crosses were examined with identical results), in each of which both parents had comparatively low growth rates, and in each case one or more rapidly growing types were obtained among the segregants. These types were not only rapid growers but had in the case of the crosses 10×2 and 15×16 a much thicker and more plentiful mycelium (see Plate VI) than either parent. As far as the investigation went these types could be described as exhibiting hybrid vigour, but no experiments were carried out to elucidate their physiological reactions. One of the rapidly growing types, type *e*, appeared among the segregants of every cross despite very marked differences between the parents. The most probable interpretation of this is that type *e* represented the original 'wild type' from which the other types had originated by gene mutation, and that a cross only proved fertile when the genotypes of the two parents were such that the wild-type

genotype was present following their union. These results suggest that it should be possible among the higher plants to select two strains which differ from a third strain (possibly a 'wild type') in that each carried a mutant character which gave a lower efficiency index, the characters being different from one another and causing the strains to have different growth indices. On crossing two such strains an F_1 generation carrying, in a heterozygous state, the factors present in the third or wild-type strain would be produced. If the factors concerned showed complete or even partial dominance, the F_1 generation would have an efficiency index greater than that of either parent and perhaps equal to that of the wild type. If, on the other hand, one of the mutants was crossed with the third strain no such increase in the growth index could be expected, the index of the F_1 generation would be either identical with that of the faster parent or intermediate between those of the parents. Should this theory be proved by experiment to be the case, it might serve to elucidate results such as those obtained by Ashby and Luckwill, where the F_1 generation was never found to have an efficiency index greater than that of the faster parent, as on the basis of the above theory this result would be expected if one of the two parents possessed no dominants or semi-dominants which the other lacked. The experiments described in this paper unfortunately throw no light on this question, as in all the crosses with the exception of 26×88 at least one parent has a growth rate which differs by only a small amount from that of the *normal* or 'wild' type.

5. SUMMARY

Eight crosses have been made between different strains of *Neurospora crassa* and the growth rates of the segregants and parents determined. Growth rates smaller than, and intermediate between, those of either parent have been found among the segregants, but in only one case has an apparent example of hybrid vigour, i.e. of a greater growth rate, been established.

This example of an apparently greater growth rate has been shown on analysis to be due to the segregant type having a response to temperature different from that of its parents. In consequence it is to be emphasized that in questions of the inheritance of physiological characters deductions based on measurements made under only one set of environmental conditions are unsatisfactory and may readily lead to wrong conclusions.

The inheritance of certain morphological characteristics and of two factors affecting the rate of spread of the fungus are recorded.

Evidence is adduced to show that cytoplasmic inheritance is demonstrable in this fungus and that it affects both morphological and physiological characteristics.

Experiments carried out by the author on *Coprinus sphaerosporus* are reviewed and are shown to indicate the presence of hybrid vigour in that fungus. A suggestion arising from this discussion indicates a possible

with Reference to Hybrid Vigour and Cytoplasmic Inheritance 129
interpretation of certain unexpected results in experiments carried out by Ashby and Luckwill.

The author has much pleasure in thanking Professor V. H. Blackman for his constant advice during these experiments and also Professor F. G. Gregory for numerous suggestions.

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The Effect on the Growth of *Sclerotinia fructigena* of Alternating Periods of Light and Darkness of Equal Length

BY

HUGH DICKSON

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With one Figure in the Text

I. INTRODUCTION

THE effect of alternating light on plants has been studied by a number of different investigators who have approached the subject from different angles (Garner and Allard, 1931; Gregory and Pearse, 1937; Portsmouth, 1937; McAlister, 1937; Pratt and Trelease, 1938; Dickson, 1938). These experiments have all been carried out with chlorophyllous plants, and it appears to have been generally assumed that the various effects obtained were due solely to the action of the alternating light on the photosynthetic mechanism of the plant. It appeared to the author as possible that at least part of the effect might be due to some action of the alternating light other than a photosynthetic one. It was therefore decided to examine the effect of alternating light and dark periods on the growth of a fungus growing in pure culture, and this paper is the outcome of these experiments.

II. EXPERIMENTAL PROCEDURE

The fungus finally selected for investigation was *Sclerotinia fructigena* Schroet, which is well known as a type which produces growth zones when subjected to the daily alternations of light and dark. In this connexion also it had already been employed by Miss Hall (1933), who studied the effects of different media on the response to the day and night changes in growth rate and form.

The fungus was cultured throughout on Petri dishes. The stock cultures were kept in darkness at 23° C. on a medium made up as follows: glucose 2 gm., ammonium chloride 1.5 gm., KH_2PO_4 1.25 gm., MgSO_4 0.75 gm., agar 20 gm., water 1 litre. On this medium the growth was thin but the rate of spread was fairly uniform over a number of days. In all experiments the fungus was grown on malt agar consisting of 2 per cent. malt and 2 per cent. agar. On this medium growth in the dark was very irregular (see below), but when exposed on the laboratory bench the fungus grew freely and formed zones with great regularity. Inocula were prepared by cutting discs of 4 mm.

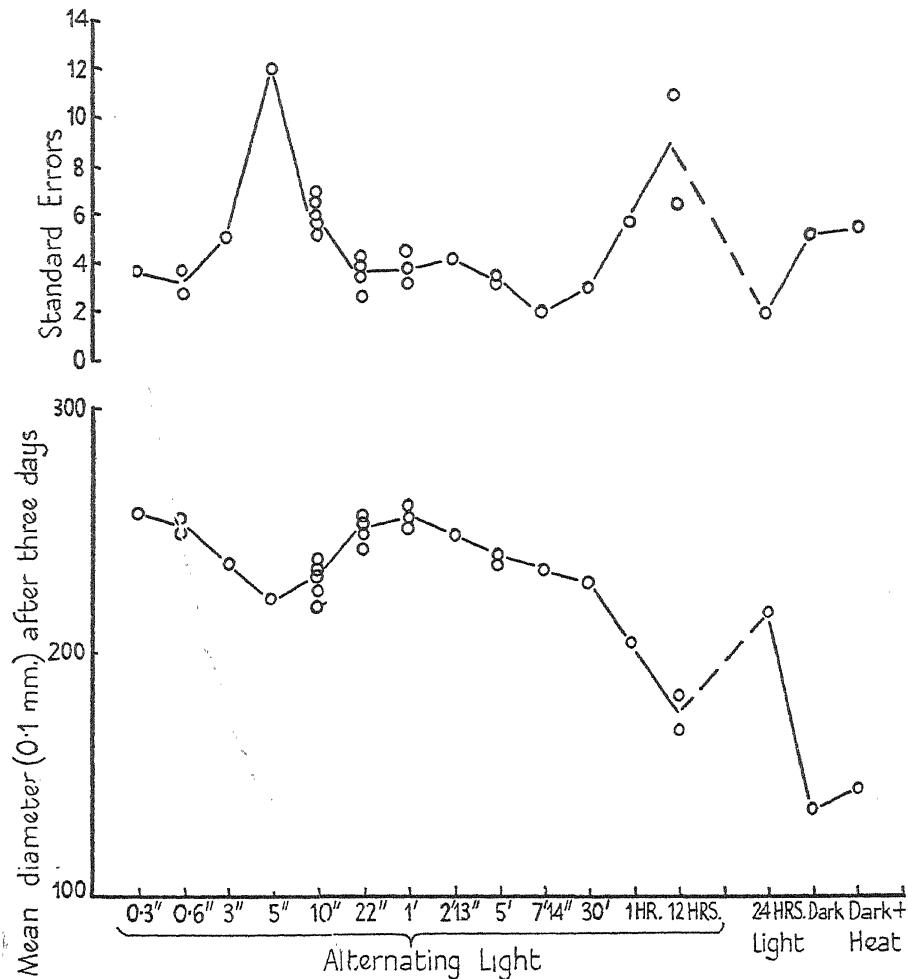
diameter from the growing edge of the stock cultures; these were then transferred to the malt agar medium and placed on the surface with the face bearing the fungus downwards. By this means a uniform area of fungal hyphae was brought into direct contact with the fresh medium, whereas where the disc had been placed fungal side up the fungus grew first down the sides of the disc of partly exhausted medium, a distance depending on the somewhat variable thickness of the disc.

During experiments the fungal cultures were placed in a water-jacketed incubator the wood door of which had been removed, the inner glass door only remaining. The interior of the incubator was divided into halves by a vertical partition, and the partition was adjusted so that light entering one part did not penetrate to the other. Black velveteen was attached to the outside of the glass door and so arranged that two 100-watt lamps illuminated the respective compartments into which the incubator had been divided, while at the same time the light from one lamp did not penetrate to the compartment illuminated by the other. Extraneous light was entirely excluded. Seven Petri dishes were arranged approximately vertically one above the other in each compartment of the incubator and facing the glass door. The incubator was maintained at 23.5° C. and appeared to be totally unaffected by such heat as was received from the lamps. The lamps were water-jacketed, a constant stream of cold water serving to remove a large proportion of the heat which they produced. Each lamp was turned on and off by a mechanism similar to that described previously by the author (1938). The following alternating periods of light and darkness were available between the two lamps: 0.3 sec., 0.6 sec., 3 sec., 5 sec., 10 sec., 22 sec., 1 min., 2 min. 13 sec., 5 min., 7 min. 14 sec., 30 min., 1 hr., and 12 hr.; the last period was controlled by a Venner time switch. Continuous light and total darkness were also used. To test the possible effects of any variation in temperature due to the switching on and off of the lamps, a source of heat, consisting of a 50-watt lamp (without a water screen) covered with a small tin, replaced one of the lamps in one experiment.

Cultures were placed in the apparatus immediately they had been inoculated and the lighting system was switched on. They remained exposed under these conditions for three days, when the diameters of the colonies were determined by means of an apparatus previously described by the author (1935). Seven plates were exposed in each half of the incubator and the mean diameters of the colonies were determined. As only two sets of plates could be exposed to different treatments at the same time the experiment was repeated fourteen times, thus giving a total of twenty-eight readings. The pairs of treatments were arranged so that the effects of short alternating periods, e.g. 0.6 sec. and 5 sec., of longer periods such as 5 min. and 7 min. 14 sec., and of long and short periods such as 30 min. and 5 sec., were determined together. Where the slope of the curve was found to change rapidly the relevant treatments were repeated several times.

III. RESULTS

The results are shown in the graph in which the lower curve shows the relationship between the mean diameter (mm. $\times 0.1$) of the colonies in three



The rate of spread in culture of *Sclerotinia fructigena* in light of different intermittencies, in continuous light, and in darkness with and without a heater. The ordinates in the lower figure represent the mean diameters expressed in 0.1 mm. obtained after three days. The upper figure shows the standard errors of these means.

days and the different alternating periods of light and darkness. The upper curve shows the variations of standard errors of these means with the different alternating periods. It is seen that from twelve-hourly alternations the growth rate increases rapidly as the periods become shorter until it reaches a maximum.

134 Dickson—Effect on the Growth of *Sclerotinia fructigena* of ; and at about one minute. It then falls to a minimum around five seconds period subsequently increases until at 0.3 second, which was the shortest period used, the rate is about as fast as at one minute alternations. Under continuous light the fungus grows more rapidly than at twelve-hourly alternation ; the more slowly than at any of the other intermittent periods determined with the exception of the minimum rate at five-seconds alternations, the rates as the seconds and in continuous light being about equal. In complete darkness the fungus grows more slowly than with any of the light treatments. The differences between the results in darkness with and without additional heat are not significant. In continuous light and at all alternating light periods, ^{by 2.} exception of twelve hours, the fungal colonies were very similar in appearance. The mycelium was evenly spread over the surface of the colony and the edge of the colony was very even. There was no zoning and the mycelial web was of medium thickness. In the case of those cultures which were grown in continuous darkness and those in twelve hours alternating light the type of growth was, however, very different. In these cases the mycelial web was very thick and close and somewhat unevenly spread over the surface and at the same time growth was very irregular so that accurate determination of the diameter was difficult. Slight zoning was apparent in colonies exposed to twelve-hourly alternations; this was, however, much less marked than in the case of similar cultures exposed on the laboratory bench, from which it is thought probable that much of the zonation seen under the latter conditions is due to temperature changes accompanying the diurnal light rhythm.

The variation of the standard errors shows a negative correlation with that of the means. This was to be expected in the case of total darkness and of twelve-hourly alternations in view of the irregularity of the colonies, which was visible on inspection. The high standard error at five-seconds alternations could not, however, have been foretold from an inspection of the cultures, as they appeared to be just as regular as those exposed to any of the other alternating periods. It is evident then from an inspection of the data that under certain lighting conditions growth is irregular and that this is negatively correlated with the growth rate.

IV. DISCUSSION

The diameter of the colony is the measure of growth used throughout this paper, and as the area is proportional to the square of the diameter it is clear that had areas been recorded then differences between the light treatments would have been even greater than those shown in the graph. In view of this it would appear that such differences as may exist between the thickness of the mycelia in the various treatments are of negligible importance and that the figures here recorded give an accurate picture of the relative rates of increase in weight of the fungus under the different lighting conditions.

A comparison of the results obtained by various workers for chlorophyllous plants.

In general chlorophyllous plants grow strongly in continuous light, less rapidly in twelve-hourly alternations, though in the cucumber the difference is very small (Portsmouth, 1937), and then progressively more slowly as the alternating periods become shorter. The growth rate reaches a minimum at about one-minute alternations and then increases again to a comparatively high level, as for example in the case of *Lemna* (Dickson, 1938, Fig. 1). In the case of the fungus, on the other hand, the growth rate is at a minimum at twelve-hour alternating periods, increases to a maximum at about the one-minute period, and then decreases. In both cases the rate is greater in continuous light than in twelve-hourly alternations. It is impossible at the present stage to draw any exact inferences from these data, but it is evident that the effect of alternating light is not solely on the photosynthetic mechanism. It would seem that alternating periods of light and darkness have some other photochemical action or have some direct effect on the protoplasm. If this is so, the growth curves obtained with a chlorophyllous plant in intermittent light represent no doubt the result of the interaction of these two effects, though of their relative importance nothing is at present known.

V. SUMMARY

Apparatus has been designed to enable *Sclerotinia fructigena* to be grown at a constant temperature and under various equal alternating periods of light and darkness ranging from alternations of 0·3 second to twelve hours. Diameters of the fungal colonies were determined after three days' growth in the apparatus.

In darkness and in twelve-hourly alternations the fungus produced an irregular colony, in the latter case showing some zonation. Under all other conditions the growth was regular. Growth was slowest in total darkness, and faster in continuous light than in twelve-hourly alternations. The rate of spread under twelve-hourly alternations was slower than with any other of the alternating treatments. With the shortening of the period of alternation the growth increased. It reached a maximum at about one-minute alternations, decreased to a minimum at five-second alternations, and subsequently rose again.

The effect of alternating light of different periodicities on the growth rate of the fungus is compared with its effect on chlorophyllous plants. It is concluded that the effect of alternating light on the growth of plants cannot be interpreted solely by its influence on the photosynthetic mechanism, but that in addition there is an action on some photochemical mechanism present in non-green plants or there is some direct effect of light on the protoplasm.

In conclusion it gives me much pleasure to express my appreciation of the interest and criticism which Professor V. H. Blackman has shown during the course of these experiments.

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Cytology of Apogamy and Apospory in *Osmunda javanica* Bl.

BY

P. C. SARBADHIKARI, D.Sc.

With Plate II and four Figures in the Text

INTRODUCTION

THE phenomenon of apogamy and apospory has for a long time been known to occur in ferns, and several writers have described its morphological characters, but our knowledge of cytological history is still inadequate. Since the discovery of apogamy by Farlow and De Bary in various species of ferns our knowledge of the phenomenon, both of apogamy and apospory, has been greatly extended by the investigations of Farmer and Digby, who have contributed much to elucidate the structural features involved with reference to chromosome cycle. The two chief modifications of the normal course of the life-history of a fern are of interest in themselves, and these phenomena have always been regarded as 'short cuts' in the life-history. In normal cases the doubling of the number of chromosomes is effected during the transition of the gametophyte to sporophyte by the addition of the chromosomes—those differences being established respectively by fertilization and by the tetrad division in the sporangium—and this double number is retained until they once become reduced to one-half in the formation of spores that introduce again the gametophyte stage.

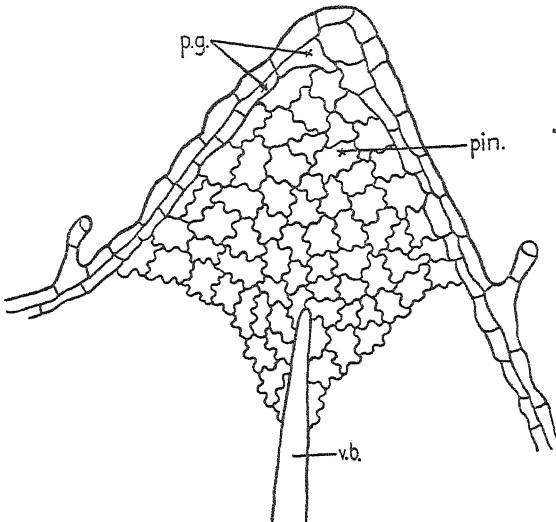
Perhaps a brief outline of the progress of investigation and opinion on the subject will serve to make the points at issue clear. The first intimation of a departure from the regular cycle of events in ferns was made by Farlow (1874), and several workers have described its morphological characters and noted the vegetative production of sporophytic buds from the cushion of the prothallus without the intervention of sexual organs. Cases of apogamy in ferns were discovered by De Bary (1878) and the subject was fully discussed. Subsequently, Druery (1884) found an apogamous fern and he demonstrated the omission of the event of spore-production, and this and other examples were investigated by Bower (1887, 1888). The subject received more general treatment later by Strasburger (1894), who noted that such sporophytic budding results merely in a repetition of the same phase of the life-cycle as that from which they arose and the budding may be repeated over and over again. This subject has again been discussed by Bower (1890) and Strasburger (1894) from the standpoint of antithetic alternation, and from

that of homologous alternation by Scott (1896). Lang (1898) observed the apogamous character of *Polypodium vulgare* var. *grandiceps* in which numerous sporophytic buds were produced from the sorus. The cellular features of apogamy were described by Farmer, Moore, and Digby (1903), and it is of interest to note that within the limits of a single, but highly variable species, almost all grades of apogamy have been encountered. Digby (1905) investigated cytologically the aposporal character of *Nephrodium pseudo-mas*. var. *cristata apospora*, where the prothallus grows directly out from the margin or from the surface of the leaf. According to Farmer (1907) certain species of *Nephrodium* (e.g. *N. pseudo-mas*, var. *polydactylum*) are known to produce prothalli on which the apogamous formation of sporophytes is of general instead of rare occurrence. In *Lastrea* and *Athyrium* it has been shown by Farmer and Digby (1907) that with the variation in form other important characters are associated. These characters not only concerned with the peculiarities associated with the particular form of apogamy exhibited by an individual variety, but they extend to minute details of cell structure. It has been shown that apospory may be initiated from the sporangium or from vegetative tissue of the leaf where a series of increasingly aberrant modes of chromosome behaviour may be seen within a single genus (Allen, 1911; Steel, 1919). It has also been recorded by the author (1936) that aposporous growth of the prothallia originates from the edge of the frond. In several genera of ferns, gametophytes may develop as buds on the sporophyte, commonly from the leaf, the margin or the meristematic tissue at the base of the sorus (Lawton, 1932).

MATERIAL AND METHODS

The material used in this investigation was collected from Blackpool, Nuwara Eliya, Ceylon. A large number of prothalli, growing wild by the side of a pool, have been examined. The abundant moisture leads to the fertilization of most of the prothalli before they have attained any considerable size. It is in districts with a suitable climate like the locality from which the specimens were collected that apogamy may be expected to occur. I began to make collections early in April in 1935 and continued to prepare material at short intervals, as long as time and the supply of material permitted, until March of the present year. Additional material was also collected for me by Mr. Abeysinghe of Ceylon University College, to whom I wish to express my thanks. As a result of previous experience with similar materials (1924, 1927), special attention was paid to fixation. Well-fixed material was obtained by momentarily immersing the material in 35 per cent. alcohol before placing it in the fixing fluid; this ensured the quick penetration of the fluid without the use of an air-pump. Ordinarily the material was left in the fixing fluid from twelve to twenty hours, washed in running water from five to twelve hours, and then slowly dehydrated by a series of graded alcohols. The fixatives used mostly were strong chromic acid, acetic alcohol, Flemming's strong

and Flemming's weak solutions. The latter gave satisfactory results in this investigation. For staining the sections Heidenhain's iron-alum-haematoxylin followed by a ground stain of orange G, and without a counter-stain, gave the best results. Breinl (safranin, methylene blue, and orange G) was also used with success. Preparations of almost every stage have been made in duplicate,



TEXT-FIG. 1. Very early stage of prothalloid growth from the edge of the frond: *pin.*, pinnule; *p.g.*, prothalloid growth; *v.b.*, vascular tissue.

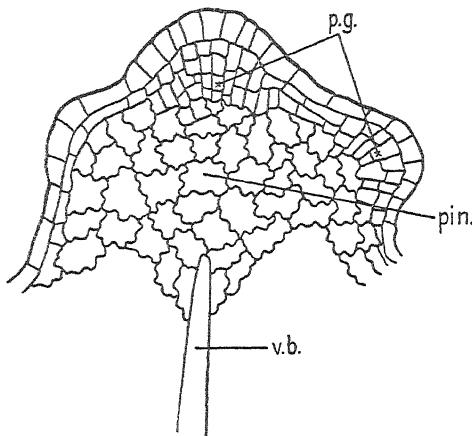
one-half of the slides stained with the triple, the other by the Heidenhain's iron-alum-haematoxylin. By this method one stain serves as a control for the other. In addition to microtome sections, observations were also made on the fronds mounted as a whole after staining with acetic carmine or borax carmine. The fronds were stained in various ways, and on the whole the triple stain of anilin-safranin, gentian violet, and orange G is very satisfactory where all stages of mitosis are concerned.

Most of the sections cut at $5-8\mu$ thick were quite useful for critical phases. In sectioning great care was taken in orientating the material so as to get a medium longitudinal section of the prothallus which should pass as nearly as possible through the median plane of the sexual organs. For some of the studies of the sporogenous cells sections were made $8-12\mu$ in thickness.

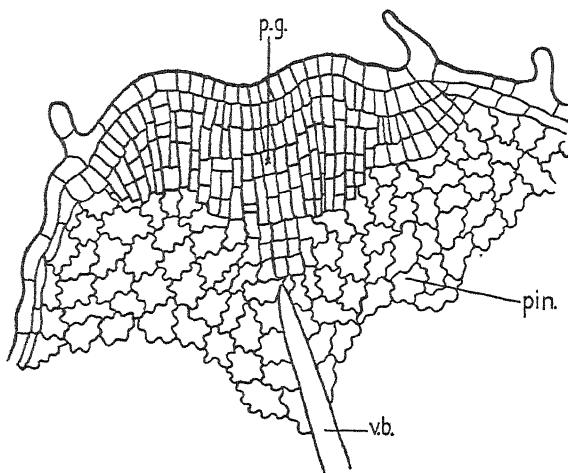
OBSERVATIONS

The prothallial growth originates either from the surface (Text-fig. 1), or more frequently from the edge of the frond (Text-fig. 2). It is at first discernible as a small outgrowth caused by the division both of the marginal cells of the leaf and of those cells lying immediately within the margin. The

marginal and apical prothallia are much more regularly heart-shaped, and resemble ordinary prothalli of delicate structure. As the growth proceeds it is distinguished as a more or less continuous sheet of delicate tissue formed



TEXT-FIG. 2. Prothalloid growth from the surface of the pinnule: *pin.*, pinnule; *p.g.*, prothalloid growth; *v.b.*, vascular tissue.



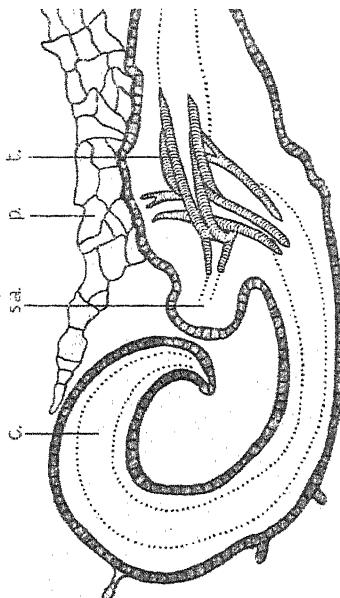
TEXT-FIG. 3. Advanced stage of prothalloid growth; it is distinguished as a more or less continuous sheet of delicate tissue formed of somewhat rectangular cells: *p.g.*, prothalloid growth; *pin.*, pinnule; *v.b.*, vascular tissue.

of somewhat rectangular cells (Text-fig. 3). The majority of the prothalli are typically regular in shape. Irregular prothalli are by no means uncommon and sometimes assume a bulbous appearance. The regular prothalli are normal in appearance except that they have no well-developed cushion. Antheridia are found frequently, even when the prothalli are comparatively

young, but archegonia have never been found on them, nor do they appear to be formed at all. In the antheridia the mother cells of the antherozoids were examined in order to test the possibility of a reduction occurring at this belated stage, but without any other result than to show definitely that no reduction occurs. The prothalli of this apogamous fern exhibit a striking feature. Nearly all the prothalli have a strand of vascular tissue extending throughout the major portion of their length (Text-figs. 2 and 3). The occurrence of these tissue elements may be considered as a simple case of apogamy. A remarkable difference exists between the cells of this plant and those of an ordinary fern. The cells and nuclei of this fern are of a distinctly smaller size. In regard to placing any great significance in the fact that in some ferns the cells of prothalli are smaller than others, it seems to me that the constancy of such difference is not sufficient to warrant any definite conclusion at present, but it is of course very suggestive that the smallness of this fern may be due to this type of cellular feature.

The appearance of the embryo is heralded by the formation of a very much localized hypertrophy situated just behind the growing point. It is often very difficult to make out the manner in which the embryo arises as a direct vegetative outgrowth, but comparison of a very large series shows that the young sporophyte is differentiated gradually from the apex and usually involves a larger or smaller number of internal cells. From numerous sections made of prothalli showing early stages in the development of embryos, it has been determined that it involves a considerable portion of both the upper and lower portions of the prothallium. The extent of the tissue belonging to the embryo can readily be determined from the appearance of the cells in a stained section. The cells of the embryo are smaller than the prothalloid cells, and their cytoplasmic contents are slightly denser. The vascular system which develops in the young embryo grows anteriorly into the leaf and posteriorly towards the root apex (Text-fig. 4).

The principal cytological interest in the prothalli centres in the number of the chromosomes, and in a comparison between the prothallus and the sporophyte in this respect. Those who are familiar with cytological work of this kind will realize that to obtain an exact number of chromosome



TEXT-FIG. 4. Longitudinal section of the embryo: *c.*, cotyledon; *p.*, prothallus; *r.*, root; *s.a.*, stem apex; *t.*, tracheides.

estimations is a task of considerable difficulty, and every care has been taken to get as near as possible to the real number.

The mean of a considerable number of actual countings in the gametophyte is 42 (Pl. II, Figs. 1, 2, and 3). This calculation is obviously too low owing to the difficulty of realizing every individual case when dealing with high numbers; forty-eight is probably nearer the actual figure. The exact number of chromosomes is not, however, of great importance, but the close approximation in the result of chromosome countings of the prothallial and sporophytic nuclear divisions undoubtedly proves that there is not reduction during the transition of the sporophyte to the gametophyte generation.

The embryo, so far as our material is concerned, invariably arises as a direct vegetative outgrowth from the prothallus, and when very young consists of cells in which the apical cells of the cotyledon, stem and root are recognizable (Text-fig. 4). The nuclear divisions have been critically worked out, and as in the prothallus, the average number of chromosomes present at each mitosis has been taken from several countings. The mean of the number obtained is forty-four, but as in the case of the nuclei of the prothallus, this is certainly too low (Pl. II, Figs. 4, 5, and 6). Of course, there is a little variation in the actual numbers obtained when dealing with chromosomes as numerous as these, but the latitude is not large. It thus appears to be clear that there is no reduction in the formation of the hypertrophy, which gives rise, without fertilization, to the apogamously formed embryo.

It is further of special interest to note here that in normal cases the doubling of the number of the chromosomes is effected during the transition of the gametophyte to sporophyte by the addition of the chromosomes belonging to the spermatozoid to those of the oosphere, and this double number is retained until they once more become reduced to one-half in the formation of the spores that introduce again the gametophyte stage of the life-cycle.

Regarding the circumstances of apogamy which have been described in this paper from the point of view of their nuclear history, it is obvious that gametophytes arise directly as outgrowths from some part of the sporophyte—whether from barren sporangia or from the tissues of the leaves—and retain the full sporophytic complement of chromosomes.

Further, out of large numbers of prothalli it was observed that there is no migration or fusion of nuclei from one prothallial cell to another.

GENERAL CONSIDERATIONS

If the studies under consideration bear out the conclusion obtained from an examination of a very large quantity of material, it would seem that, in the fern in question, there is no reduction of the chromosomes on passing from the sporophyte to the gametophyte. There is no migration or fusion of nuclei in the cells of the prothallium of this fern. The embryo arises apogamously as a direct vegetative outgrowth from the prothallus. As a matter of fact, both the sporophyte and the apogamous gametophyte arising from it

have the unreduced number of chromosomes. From this it is evident that apospory is balanced by apogamy, the chromosome number remaining the same throughout the life-cycle.

Every stage of the nuclear division has been critically studied both in the developing sporangia and in the prothallial outgrowth, and the result is to confirm the statement to the effect that the transition from the sporophyte to the gametophyte in this fern is attended by no reduction in the number of the chromosomes. Further, as a result of comparison of the chromosomes of the sporophytic tissue in the embryo with the young cells of older prothallia, the statement is confirmed that there is no change in the number of chromosomes which marks the transition from one stage in the life-history to another. Consequently, it might be said that the embryos of the 'gametophytes' of this fern arise apogamously or, in other words, they are formed without fertilization from cells or tissues that already possess the full complement of 'sporophytic' chromosomes which have persisted unchanged through that cycle of life-history which is generally known as the gametophyte. In this case the life-cycle shows the two generations as usual but without any changes in the number of chromosomes, that is, the sporophytic number of chromosomes is carried through to the gametophyte. It seems to be worthy of note here that when apospory has occurred in the life-cycle then an apogamous development seems the only way of continuing the life-history.

Within recent years a certain amount of cytological evidence has thrown some light on the facts and meaning of the sexual fusion and correlative process of meiosis. But the cyclically recurring events, generally known as alternation of generations, are still vague owing to lack of convincing evidence. Considerations of this kind have attracted the writer to the study of apospory and apogamy with special reference to the cytological aspects of the problem.

The evidence afforded by the material on which the study of these prothalli has been based leads to the conclusion that no necessary correlation exists between the two phenomena, namely, the periodic reduction in the number of chromosomes and the alternation of generations. The doubling of the chromosomes receives an explanation strictly analogous to that afforded by the normal fusion of the oosphere and spermatozoid. But instead of one cell only serving as the starting point for the new generation, a number of such units loosely co-operate to produce it. From the above study it might be suggested that both the theories of antithetic and homologous alternation afford possible explanations of the facts of the normal and abnormal life-history of such a group as the ferns.

SUMMARY

The results obtained from the study of *Osmunda javanica* may be summarized as follows:

1. Aposporous growth of the prothalli originates either from the surface, or more frequently from the edge of the frond.

2. Nearly all the prothalli have a strand of vascular tissue extending throughout the major portion of their length.
3. The gametophyte never produces archegonia, but antheridia are found frequently which develop normal antherozoids.
4. There is no migration or fusion of nuclei from one prothallial cell to another.
5. The embryo arises apogamously as a direct vegetative outgrowth of the prothallus. The apical cell of the leaf is first formed, and then that of the root, and later that of the stem.
6. The transition from the sporophyte to the gametophyte is attended by no reduction in the number of the chromosomes.
7. When apospory and with it the omission of meiosis has occurred in the life-cycle, then an apogamous development seems the only way of continuing the life-history.

In conclusion, I should like to take this opportunity of acknowledging my indebtedness to Sir John Farmer for the helpful criticism and advice that he has so kindly given me. My sincere thanks are due to Professor V. H. Blackman for his kind help with the manuscript.

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EXPLANATION OF PLATE II

Illustrating Dr. Sarbadhikari's paper on 'Cytology of Apogamy and Apospory in *Osmunda javanica* Bl.'

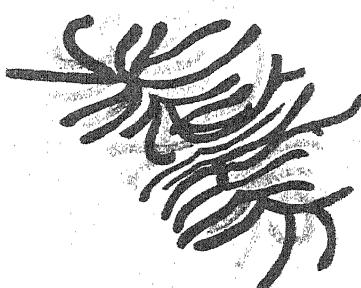
All the figures were drawn with the aid of an Abbe camera lucida under a lens 2 mm. apochr. N.A. 1·4 and comp. ocular 18 at a magnification of about 2,250.

Figs. 1-3. Nuclear divisions in the prothallus.

Figs. 4-6. Nuclear divisions in the embryo.



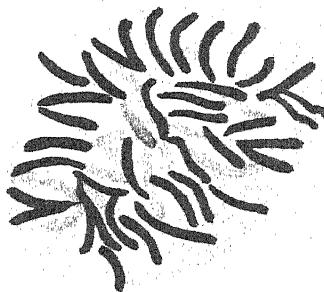
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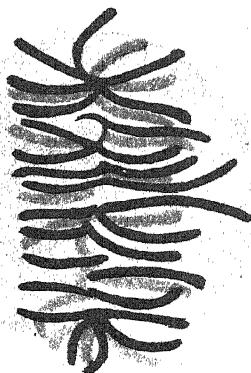
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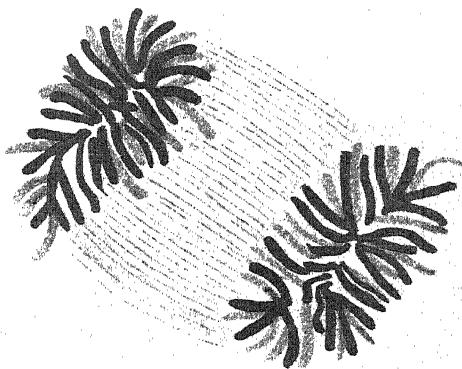
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SARBADHIKARI — APOGAMY AND APOSPORY IN OSMUNDA.

An Apparatus for the Study of the Oxygen, Salt, and Water Uptake of Various Zones of the Root, with some Preliminary Results with *Vicia Faba*

BY

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AND

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With Plate III and one Figure in the Text

INTRODUCTION

VARIOUS methods have in the past been used to establish experimentally the variation in uptake of water by different zones of the root. Less attention has been devoted to the study of the variation in metabolic activity along the root, or the variation in salt uptake in different regions. In this paper a method is described which permits of the study, if necessarily simultaneously, of these activities in varying zones of a single root.

In so far as water uptake is concerned, the earlier experiments have been reviewed by Popesco (1926), Ursprung and Blum (1928), Höhn (1934), and Sierp and Brewig (1935). The earlier work depended on indirect methods, by vital staining or microchemical tests (Popesco). Ursprung and Blum dismiss this work on the ground that the staining reactions of the various zones bear no relation to water-uptake rate. Ursprung and Blum (1928) employed the method of suction-pressure determination, and their results therefore suffer from all the disadvantages of the method and its indirect approach to the problem of estimating velocities of uptake.

The potometric method had been used by early investigators and the details of technique employed are discussed at some length by Höhn (1934). The main difficulty lies in the isolation of different zones of the root. Popesco (1926) attempted to use cocoa-butter, but Höhn, who tried this method, found it unsatisfactory, since watertight union with the root could not be secured.

Chodat (quoted by Höhn) appears to have first used oil for this purpose.

¹ This work was carried out while the second author held a Beit Research Fellowship at the Imperial College of Science and Technology.

The later work of Höhn (1926), Sierp and Brewig (1935), Rosene (1937), and the present work all employ the potometric method and differ in the manner in which the difficulty of isolating the root zones has been met.

Höhn failed in his attempt to seal roots into potometers either by 'Plastelin' or Leick's gum (Leick 1928). He therefore abandoned all attempts to isolate various zones, and used instead roots after varying times of growth and thus virtually consisting of different zones.

Sierp and Brewig (1935) attached on the same root a series of micropotometers covering adjacent segments. The joint between potometer and root surface was secured by wrapping with thread impregnated with lanolin.

Rosene (1937) employed potometers consisting of calibrated capillary tubes of uniform bore. One end of each tube was ground down on opposite sides and a hole bored through the capillary to permit passage of the root. A number of such potometers were threaded on a single root. This method, which is simple in conception, is clearly capable of yielding precise quantitative data.

The results obtained for water uptake by these various investigators are not in agreement, partly attributable to the various plant materials used and without doubt in some cases to deficiencies in the technique.

With regard to the variation in ion uptake in different zones of the root, Prevot and Steward (1936) have recently shown that there is a longitudinal gradient of bromide absorption by roots of barley, broad bean, and cotton, the root segment nearest the apex absorbing more than those more remote. These authors discuss the metabolic and developmental factors involved in this longitudinal gradation of salt accumulation and conclude from the known relation between respiration and salt absorption that it is an inevitable consequence of the progressive development of cells from the root apex.

No quantitative measurements of the gradation of metabolic activity have as yet been reported. Lund and Kenyon (1927) claim, however, that their electrical measurements can be correlated with oxidation and indicate the gradation of metabolic activity along the root. These investigators also determined the approximate oxygen requirements of different regions of the living root by the use of methylene blue. This dye which stains roots is more readily reduced by the active growing regions of the root (see also Prevot and Steward). By the use of phenol red they also claim to have shown that there was a greater production of carbon dioxide at the root-tip.

From a survey of the literature cited it might be concluded that the rate of water absorption increases in a proximal direction from the root-tip and that the distance from the root-tip at which there is a maximum rate of absorption is dependent upon the type and age of plant used, and in addition upon many environmental factors. The results of Prevot and Steward suggest a relation between gradation of salt absorption and metabolic activity, but there appears to be no direct relation between the gradients of salt and of water absorption.

METHODS AND APPARATUS

Primary roots of *Vicia Faba* were used in all experiments. The seeds were germinated in test-tubes containing moist sphagnum moss and were grown in a chamber maintained at $22^{\circ}\text{C.} \pm 1^{\circ}$, illuminated for 16 hours daily with four 100-watt Osram lamps. By the time the first leaf had expanded, the primary root was approximately 8 cm. long and bore a few laterals at the proximal end. The length of the root and the number of laterals were found to depend greatly on environmental conditions which were kept standard as stated. The apical 5 cm. of the root, free from laterals, was enclosed in the apparatus.

The method of dividing the root into compartments for the measurement of absorption and respiration is shown in the Text-figure and Plate III. The plate figures a seedling which had been growing in the apparatus for four days. The healthy condition of the root is shown by the growth of laterals into the upper two compartments and also the elongation of the apical segment, which at the time of assembling protruded only half-way into the lowest compartment.

The apparatus consisted of five cylindrical glass cells, approximately 1 cm. in height, to opposite sides of which were fused two fine-bore glass tubes. The ends of the cylinders were ground flat and so fitted evenly one upon the other. These cells were held in place in a metal stand, the top of which was movable and could be fixed firmly into position by two screw nuts. The seedling was held in a glass container, into the bottom of which was sealed a short piece of glass tubing, which in turn was cemented into a hole in the movable metal clamp. The root passed through this hole into the cells below and each section was made airtight in the manner shown in the Text-figure (p. 150).

The diaphragms separating the chambers consisted of pieces of sheet rubber into the centre of which a very short length of valve-tubing was sealed with rubber solution. In order to fix the diaphragm in position its central tube was first pushed over the end of a tapered piece of glass tubing just large enough to fit over the region of the root at which the diaphragm was to be placed (cf. Gregory, 1938). When the glass tube was in position over the root, the diaphragm was slipped off the piece of tubing and so made an airtight seal with the surface of the root. The size of collar used was determined by the thickness of the root and provided a proper choice was made there was very little squeezing of the root tissues. The diaphragms were then stretched between the cells and held in place by the flat ground glass rims of the cells, which were tightly clamped together.

Oxygen and nitrate absorption were estimated by allowing 10 c.c. of water or culture solution to pass over the root segments at approximately $\frac{1}{2}$ c.c. an hour and analysing the solution before and after contact with the root. All experiments were carried out at 25°C. and the water for chemical

determinations was collected under liquid paraffin in 10 c.c. graduated measuring cylinders. The micro-Winkler method of Krogh (1935) was employed, using a 10-c.c. syringe pipette. Water uptake was measured by closing one

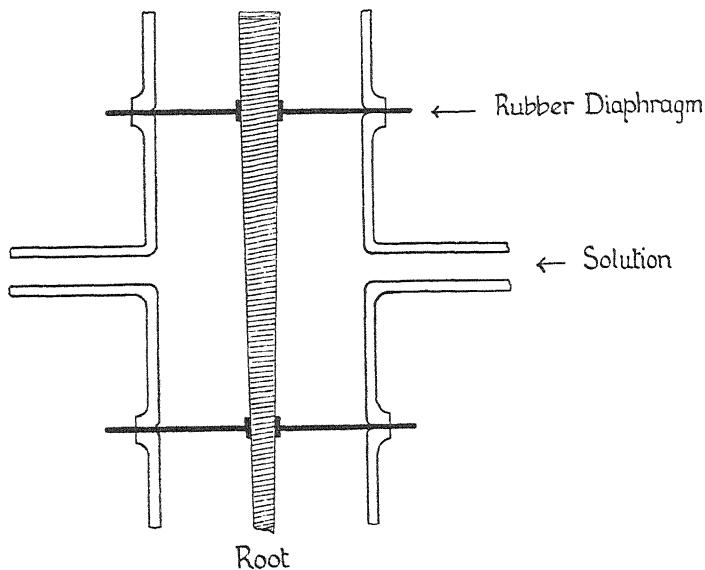


Figure showing method of fixing root segments into separate chambers.

side arm of the glass cell and observing the movement of water in a micro-potometer attached to the opposite arm.

PRELIMINARY EXPERIMENTAL RESULTS

Oxygen uptake.

The results of a preliminary experiment are presented in Table I. Two consecutive measurements of oxygen uptake were made over 18- and 11-hour periods. The results are expressed as total oxygen uptake per root segment and also on the basis of root-surface area, measured by means of a travelling microscope.

On the basis of surface area the results show that oxygen uptake was highest in the segment no. 5, containing the tip and decreased in segments 4 and 3 in both experiments. The high value for segment 1, experiment 1, was probably due to the presence of a root lateral. At the end of experiment 2 there was a definite rupture in the cortex and gas from the tissue had half filled the glass cell of segment 1. It was noticed in all experiments that gas was forced out of the root in considerable quantities slightly before and during the emergence of a root lateral. The nature of this gas was not determined, but it was noticed that apparently it did not affect the oxygen concentration of the external solution.

Several experiments were carried out in this manner and all gave similar results. In the majority of experiments the total uptake of oxygen was highest in the segment containing the root apex and decreased in a proximal direction. This was true of all experiments when the results were expressed on the basis of root surface area.

TABLE I

Uptake of Oxygen by Adjacent Root Segments

Root segm.	Surface area of Segment (mm. ²)	O ₂ in 10 c.c. H ₂ O (c.c.)	O ₂ used (c.c.)	O ₂ used per mm. ² (c.c. × 10 ⁶)	O ₂ used per mm. ² per hr. (c.c. × 10 ⁶)
Expt. 1 (18 hrs.)					
1	113	0.0262	0.0109	96	5.3
2	142	0.0267	0.0104	73	4.1
3	78	0.0304	0.0067	86	4.8
4	73	0.0279	0.0092	126	7.0
5 (Apex)	21	0.0301	0.0070	333	18.5
Original H ₂ O		0.0501			
Expt. 2 (11 hrs.)					
1	113	(Gas)	—	—	—
2	142	0.0355	0.0011	7.7	0.7
3	78	0.0302	0.0004	5.1	0.5
4	73	0.0335	0.0031	42	3.8
5 (Apex)	25	0.0323	0.0043	171	15.5
Original H ₂ O		0.0496	—	—	—

Later experiments showed, however, that the rubber diaphragms were also taking up oxygen either by absorption or oxidation. In Table I it was assumed that each diaphragm used the same amount of oxygen (0.0130 c.c.) over the experimental period. Experiment 2 did not last as long as experiment 1, and the use of the same correction factor for both experiments probably accounts for the apparent lower uptake rates of experiment 2.

Various methods were tried to prevent this action of the rubber—such as painting, covering with gold leaf, using synthetic rubber—but all proved unsuccessful. A high grade of medical rubber (dental dam rubber), previously boiled for one hour, took up the least oxygen of any tested and was used in all the following experiments.

In these experiments the roots were sealed into only two or three of the top sections of the apparatus, and the lower sections, with the rubber diaphragms in place, were filled with water to serve as blanks. The results are presented in Tables II, III, and IV. In experiment 3 (Table II) the oxygen absorbed by the rubber diaphragms in the blank cells was approximately equal and the uptake of oxygen per surface area was highest in the segment containing the root tip. Experiment 4 (Table III) shows the same effect, although in this case there was a much greater difference between the uptake by the apical and adjoining segments. Table IV presents the results of two

experiments carried out on the same root, the first experiment with the leaves illuminated for $18\frac{1}{2}$ hours, and the second experiment with the leaves in the dark for 16 hours. The results again show a very marked gradation in oxygen uptake in a proximal direction from the tip and a higher uptake when the leaves were in the light.

TABLE II
Oxygen Uptake by Root Segments (Experiment 3)

Root segm.	Length (mm.)	Area Segment (mm. ²)	O ₂ in 10 c.c. H ₂ O (c.c.)	O ₂ used (c.c.)	O ₂ used per mm. ² (c.c. $\times 10^6$)	O ₂ used per hr. per mm. ² (c.c. $\times 10^6$)
1	13.0	92	0.0335	0.0044	48	2.4
2	14.5	73	0.0280	0.0099	136	6.8
3 (Apex)	16.0	50	0.0297	0.0082	164	8.2
4	—	—	0.0374	0.0109	—	—
5	—	—	0.0384		—	—
	Original H ₂ O		0.0488	—	—	—

TABLE III
Oxygen Uptake by Root Segments (Experiment 4)

Root segm.	Length (mm.)	Area Segment (mm. ²)	O ₂ in 10 c.c. H ₂ O (c.c.)	O ₂ used (c.c.)	O ₂ used per mm. ² (c.c. $\times 10^6$)	O ₂ used per hour per mm. ² (c.c. $\times 10^6$)
1	14.0	110	0.0318	0.0051	46	1.7
2 (Apex)	12.0	35	0.0236	0.0133	371	13.6
3	—	—	0.0369	0.0119	—	—
	Original H ₂ O		0.0488	—	—	—

TABLE IV
Oxygen Uptake by Root Segments of Plants in Light and Darkness

Root segm.	Length (mm.)	Area Segment (mm. ²)	O ₂ in 10 c.c. H ₂ O (c.c.)	O ₂ used (c.c.)	O ₂ used per mm. ² (c.c. $\times 10^6$)	O ₂ used per mm. ² (c.c. $\times 10^6$)
<i>Exp. 5. Leaves in light (18.5 hrs.)</i>						
1	12.5	108	0.0219	0.0181	167	9.0
2	12.0	79	0.0209	0.0191	242	13.8
3 (Apex)	10.8	39	0.0143	0.0257	659	35.6
4	—	—	0.0406	0.0149	—	—
5	—	—	0.0394		—	—
	Original H ₂ O		0.0549	—	—	—
<i>Exp. 6. Leaves in dark (16 hrs.)</i>						
1	12.5	108	0.0481	0.0069	83	5.2
2	12.0	79	0.0467	0.0083	105	6.4
3 (Apex)	10.8	39	0.0399	0.0151	387	24.2
4	—	—	0.0561	0.0142	—	—
5	—	—	0.0540		—	—
	Original H ₂ O		0.0697	—	—	—

Nitrogen absorption.

Only one preliminary experiment was carried out in which nitrogen absorption was followed. The seedling of broad bean contains large reserves of

nutrients in the cotyledons, and it would appear that it absorbs salts very slowly from the culture solution (8 milli-equivalents nitrate per litre). Nitrogen was estimated by the micro-Kjeldahl method, using the apparatus of Parnas and Wagner as described by Pregl (1920). Nitrate was included by an adaptation to the micro-scale of the reduced iron method of Pucher, Leavenworth, and Vickery (1930).

TABLE V
Nitrogen Absorption by Root Segments

Error of nitrogen estimation. ± 0.003 mg. N.

Root segm.	Segment (mm. ²)	N. absorbed from 10 c.c. solution (mg.)	N. absorbed per mm. ² (mg. $\times 10^6$)	N. absorbed per mm. ² per hour (mg. $\times 10^6$)
1*	113	0.0435	385	17
2	142	0.0203	143	6
3	78	0.0522	669	29
4	73	0.0551	755	33
5	36	0.0595	1653	72

* Root lateral present. Area approximate only.

TABLE VI
Water Uptake by Adjacent Root Segments (c.c. $\times 10^4$)

			Root segm. (mm. from apex)
Experiment 1	.	.	45-37. 35-27. 25-15. 13-0.
July 2. 10.35-11.00	:	:	0.827 0.279 0 0
July 3. 3.30-4.00	:	:	1.18 0.451 0 0
Experiment 2	.	.	40-30. 27-17. 15-5. 4-0.
July 12. 10.20-12.30, 1.30-3.30	:	:	0 0.118 0.147 0.200
July 13. 11.00-12.00	:	:	0.084 0.019 0.078 0.071
Experiment 3	.	.	58-45. 43-30. 28-16. 14-0.
July 15. 12.20-1.30	:	:	0.028 0.041 0.030 0.077

In Table V are presented the results of an experiment which measured the absorption of nitrate nitrogen over a 23-hour period. The experiment was carried out on a root previously used for oxygen absorption measurements (Table I) and a root lateral was emerging in root segment 1. The results again show that the highest absorption was in the segment containing the root apex and that it decreased in a proximal direction. Segment 1, however, absorbed more nitrogen than segment 2. This was undoubtedly due to the presence of the root lateral.

Water uptake.

A few preliminary experiments were carried out to investigate water uptake of different zones and a sample of the results is presented in Table VI. The results show extreme variability. Even during the course of an experiment on the same root it was found that the relative rate of absorption between segments varied.

The results do show that all segments were capable of taking up water, but it is impossible to compare these results with the conclusions of the earlier workers because the apical segment was large enough to include practically the whole extending zone of the root.

SUMMARY

An apparatus is described by means of which it is possible to measure quantitatively the oxygen, salt uptake, and water uptake of adjacent segments of single intact root.

It is shown in *Vicia Faba* that the metabolic activity and nitrogen uptake are highest in the apical segment and decrease in those more remote.

No consistent results were obtained for differences in the water uptake of different segments.

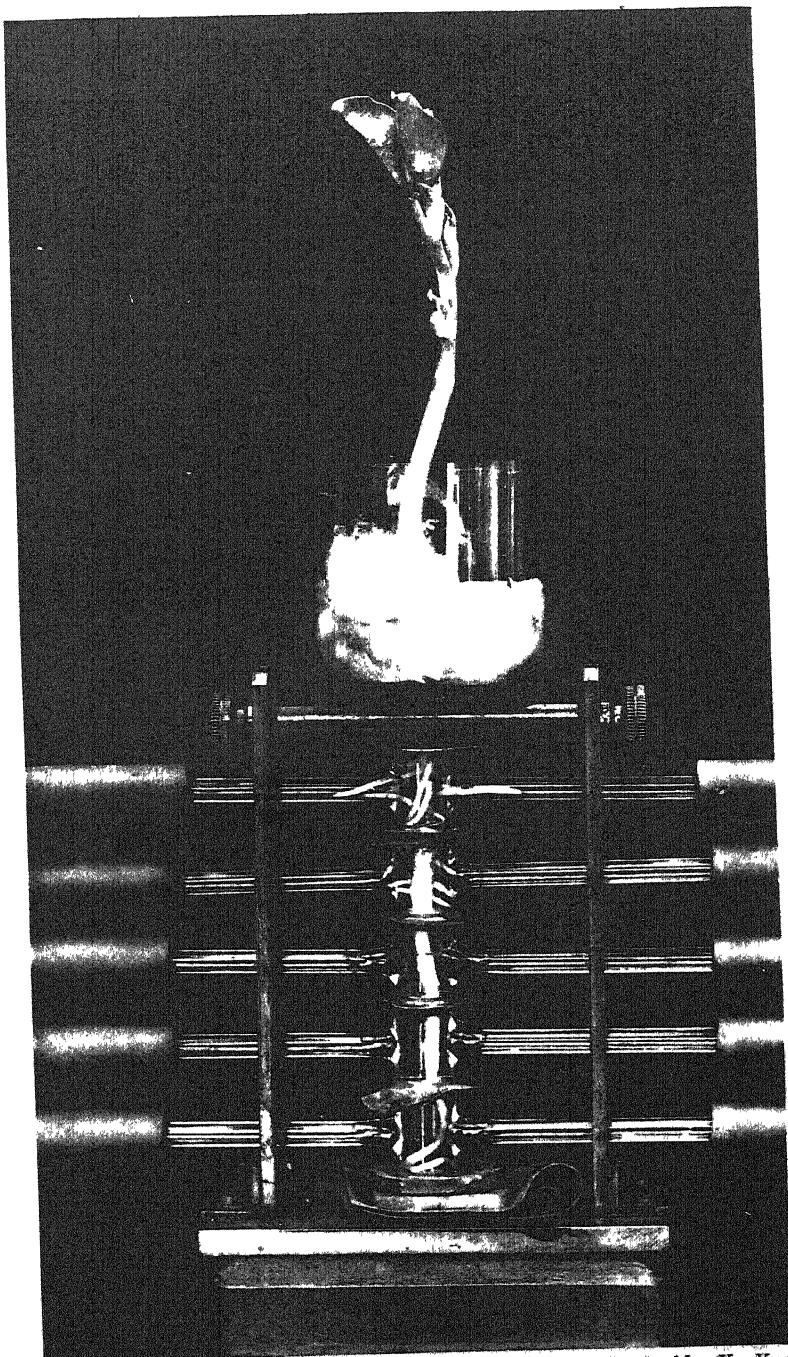
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EXPLANATION OF PLATE III

Illustrating Prof. F. G. Gregory's and Dr. H. K. Woodford's paper 'An Apparatus for the Study of the Distribution of Oxygen, Salt, and Water Uptake of Various Zones of the Root, with some Preliminary Results with *Vicia Faba*'

Apparatus for determining the uptake of oxygen, salt, and water in different zones of the root. The plant root (*Vicia Faba*) is enclosed in a series of glass cells separated by thin sheets of rubber. These diaphragms carry in the centre a very short length of valve-rubber tubing which fits closely round the root. (For details see diagram and text.) The glass cells are held together in a metal stand, as shown. The side-tubes of the cells permit the passage of water or nutrients over separate zones of the root, and, by collecting the fluid under liquid paraffin, oxygen determination and chemical analyses can be performed.



Huth, Stubbs X, Kent.

Plant Injection as a Physiological Method

BY

W. A. ROACH

(*East Malling Research Station, Kent*)

With Plate IV and thirty-four Figures in the Text

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INTRODUCTION

Definition of Injection

THE term 'injection' is used in the medical, veterinary, and zoological sciences in its correct etymological sense, viz. to indicate the forcing of a fluid into the body. Botanists have used the term in a wider sense. Thus, when the intercellular spaces in a plant become filled with sap in place of air, the tissue is said to be injected. When a liquid is introduced into a plant through a cut or a hole in one of its organs this also is called injection, even when little or no force is used. Again, a plant may be injected in this sense

without recourse to wounding of any kind. Thus, if a leafy stem be bent down under the surface of water in a convenient container, water may pass in through the leaves and the tissues become injected. Finally, if substances in a solid form are introduced into holes or cuts in a plant organ, such treatment has in recent years been termed 'solid injection'. In the present paper the term 'injection' will be used to indicate the introduction by various methods of liquids and solutions into plant organs, whether under pressure or not, and their spread therein; solid 'injection', however, is not dealt with in it at any length.

History

Early work.

Tree injection has been practised since very early times. Ibn-Al-Awam,¹ writing in the twelfth century, quoted from a work written by Hadj de Granade in 1158 which described methods for imparting perfumes, flavours, and medicinal qualities to fruits, and a yellow or blue colour to roses. The methods consisted either of splitting the root or shoot, removing the pith and introducing suitable substances into the pith cavity, or in inserting such materials between the carefully lifted bark and the wood. The substances included musk, cloves, saffron, &c., in finely pulverized form. It is clear from this that solid injection was tried at least as early as the twelfth century.

No further reference to plant injection can be traced until the fifteenth century, when Leonardo da Vinci (1490) made an entry in his note books of which the following is a translation:

'Boring a hole in a tree with a gimlet and inserting in it arsenic and realgar refined by sublimation and dissolved in boiling water is capable of making the fruit of the tree poisonous or of making it wither. The hole must be large and must go right through to the pith and should be made when the fruits are ripening. The poisonous liquid should be squirted in with a syringe and the hole should be plugged tight with wood. The operation can also be carried out when the sap is rising in the trees.'

The wording of this entry suggests that Leonardo da Vinci carried out systematic experiments and that the resulting method was devised for practical use. This is perhaps the earliest reference to liquid injection.

Methods similar to those described by Hadj de Granade were published anonymously in 1602 in 'The Orchard and Garden' as follows (Anon, 1602):

'... well smelling and spiced fruit. Cleave a tree asunder or a branch of a fruitful tree, to the heart or pith, and cut a piece out of it, and put therein powdered spices, or whatsoever you will, or what colour you will desire, and tie a barke hard about it and anoint it with lome and oxe dung, and the fruit will get both the savour and colour, according to the spice you have put in it.'

¹ The writer is indebted to Prof. R. A. Fisher for this reference and to Mr. D. Akenhead for the translation from the Italian.

² The writer is indebted to Mr. R. M. Greenslade for this reference.

'How sower fruits be made sweet.'

'Which tree beareth sower fruits, in the same pierce a hole a foot or somewhat lesse above the root and fill that with honey, and stop the hole with a haw-thorne braunch, and the fruit will be sweet.'

'How the wormes are to be killed, if they be already grown into the tree.'

'If you will kill the wormes, which grow into the tree, take pepper, laurell, and incense, and mingle all well together with good wine, and pierce a hole into the tree downward, till to the pith or heart of the tree, and poure this mixture into it, and stop it with a hawthorne, and the wormes will die.'

As stated above, these passages bear a general resemblance to that in Ibn-Al-Awam's book, but the idea of killing 'wormes', i.e. wood-boring insects, is fresh. This and other minor differences suggest that during the intervening five centuries the possibilities of solid injection were at least thought of, if not tested experimentally. The descriptive sub-title to this later work, 'Gathered from the Dutch and French', and the fact that it was published in London, suggest that the idea of plant injection may have been widely distributed.

A further development of the idea is contained in J. M. Wilson's 'The Rural Cyclopaedia' (Wilson, 1765):

'A curious method of destroying insects on trees and shrubs, by means of mercury, was invented in 1765 by a person in Hereford, and was speedily adopted by members of the Society for the Encouragement of Art. The inventor pierced the branch of a plum-tree slopingly with an awl through the bark and part of the wood, but not to the pith, and poured into the hole a drop or two of mercury, and stopped it up with a small wooden plug; and he found that, on the next day, all the insects dropped off from that branch, and, in a day or two more, from all the other branches, and that the tree continued in full vigour, and thrrove well throughout the season.'

These early attempts, though interesting historically, appear to have been the results of more or less inspired guesses, and the efficacy of most of the methods used may well be doubted; but the idea behind them, like that behind modern injection work, was to improve the health of the plant or to bestow on it special qualities without either killing or even unduly damaging it.

The knowledge available at the time hardly allowed these early experimenters to attain their aims, and no further attempt seems to have been recorded for about a hundred years. During the nineteenth century, developments occurred in more than one branch of science which supplied much of the information necessary for new and more successful injection work; and as soon as this had become available a wave of injection experimentation swept more or less simultaneously through Russia, Germany, America, and France. This new and more scientific development of plant injection was mainly the indirect result of the attempts of plant physiologists to elucidate the cause of the ascent of sap in trees; and in spite of the lack in plants of any circulatory system comparable to that for blood in animals, further impetus

was probably given by the striking results obtained on animals and man by medical workers towards the end of the century through the use of injection methods. The first fact of importance in the scientific development of plant injection was recorded by Magnol (1709), namely, that certain coloured solutions are absorbed by the cut stems of twigs immersed in them, and pass up the stems into the leaves and flowers, the path followed being clearly revealed by the colour. Magnol actually used the method for tracing the path followed by the transpiration stream, a matter which will not be considered in the present paper.

Nearly a century later, Meyer (1808), by cutting off the top of a small tree and submerging the stump in a dye solution proved that the dye penetrated the roots, reaching all except the thinnest rootlets.

Hartig (1853) seems to have been the first to lead liquid from a reservoir into a hole made in a tree, in this way adapting da Vinci's method for injecting large volumes of liquid.

McNab (1871, 1875) proves spectroscopically by experiments similar to those of Magnol that metals ascended amputated living branches at different rates when their cut ends were placed in solutions of metallic salts, viz. lithium 12·8, thallium 7·5, and caesium 3·3 in. in an hour. A few years later Pfitzer (1877) by similar experiments showed that water travels more quickly than the lithium salt dissolved in it, and he made the further important observation that the rate of travel of the injected lithium solution was greater than could be accounted for by mere diffusion.

Sachs (1878), in experiments similar to those of Magnol, McNab, and Pfitzer, used both dyes and simple inorganic salts. He noted that whereas a lithium salt travelled up the stem nearly as rapidly as the water in which it was dissolved, substances which dyed the cell walls rose much more slowly. This slowing down of the movement of many dyes as they pass through living or dead plant tissues or capillary spaces, such as those in blotting-paper, was studied in great detail by Goppelsroeder (1889, 1901), and some of the methods to be mentioned later for selecting the most suitable dyes for injection experiments were first described by him.

Work on plant injection published after 1880.

It will be noticed that most of the injection experiments so far alluded to were carried out in the hope that the substance injected would merely add its own properties to those of the plant or part of the plant injected. For instance, cloves were to give their flavour to the apples borne on the injected tree and plant dyes their colour to the flowers borne on the injected tree. But after plant physiologists had used injection methods for some time in the attempt to study the ascent of sap they began to realize the possibilities of introducing nutrient substances by this method. Thus Sachs, who had published a paper on the ascent of sap in 1878, in 1886 gave an account of experiments in which ferrous sulphate and ferric chloride solutions were

injected into chlorotic Acacia trees. His method was a simplified form of the one employed by Hartig. Sachs noticed that only the leaves on branches vertically above the hole became green.

From this time onward there was a comparatively rapid development of ideas both in regard to the actual methods used and as to the purposes for which they could be employed; and in this development national trend of thought can be recognized, which will now be briefly reviewed.

Russian work. An idea developed by Shevyrev (1894, 1903)¹ had a great influence on the development of injection methods, but how far it was based on fact cannot even now be assessed. This was that the access of air to the exposed tissues must be rigidly prevented, and Shevyrev suggested that neglect of this precaution was the cause of the failure of others to induce penetration of liquid into tissues other than those directly above the cut wood vessels (but see p. 211). The two methods he devised to fulfil this purpose have been used by a number of later workers.

Roth (1896), in work carried out in Germany apparently in ignorance of Shevyrev's paper, far from excluding air, left his injection hole exposed to the air for thirty-six hours before admitting the liquid, and he found that absorption was remarkably rapid. Shevyrev's chief experiments were carried out mainly with dyes to establish general principles.

According to Jaczewski (1910), Nicolaev-Tzygankov (1898) treated chlorotic trees successfully by introducing powdered ferrous sulphate into holes drilled to the centre of the stem. Reshko (1903) used the same method in 1901 on 1,000 chlorotic trees, but the distribution of the salt was irregular, with the result that a number of branches escaped treatment.

Mokrzecki (Mokrjetsky), a Pole working in the Crimea (1903a, b; 1904a, b, c)² also carried out extensive injection experiments. In his paper, published in German, he called the treatment, 'Die innere Therapie der Pflanzen'. He employed both a slight modification of Shevyrev's method for liquids and the one used by Nicolaev-Tzygankov (commonly named after Mokrzecki) for solids. He injected more than 500 trees by Shevyrev's method, employing solutions of nutrients varying in concentrations from 0·01 to 0·1 per cent. until no more was absorbed. He treated chlorotic trees with either 12 gm. of dry ferrous sulphate (for a tree 16–25 cm. in diameter) or with 0·05–0·25 per cent. ferrous sulphate solution. The green colour began to appear four days after injection and no trace of chlorosis was left after ten days. Three weeks later the foliage was dark, glossy, and healthy. He also used powdered, dry iron pyrophosphate for the same purpose. This treatment and the injection of Knop's and other inorganic nutrient solutions appeared to control infestation with the scale insects *Diaspis fallax* on pears and *Mytilaspis pomorum*.

¹ The writer is indebted to Mr. H. P. Gould, U.S. Dept. Agriculture, Washington, for locating them, and to Mr. C. Audrey Richards of Madison, Wisconsin, for supplying a translation of these two papers.

² The writer is indebted to Dr. J. Majewsky for some of these references and for checking the account of Mokrzecki's work.

on apples. Gummosis of apple, pear, and other trees was cured by injections of 1 per cent. salicylic acid solution. He also attempted to control bark beetles and other insects by similar means. He illustrates a tree that was treated with iron pyrophosphate on one side only, as a result of which the treated side became healthy, in marked contrast to the other side which remained chlorotic.

In 1903 Dementiev (1914), in ignorance of the work of his fellow countrymen and of Goff (1897), injected various poisonous solutions through cuts made in roots, leaf-stalks, and leaves of plants in attempts to make them distasteful to, or unsuitable food for, insects. His final plan resembled Goff's root-stump method. He found that the rate of uptake could be accelerated by increasing the injection pressure and that pressures up to 6 atmospheres could safely be employed on fruit trees.

French work. In France, also, injection methods were being developed about this period. Ray (1901) injected liquids, including solutions derived from micro-organisms, into leaves through capillary tubes, his aim being to make the leaves immune from disease. Simon (1906) reported injection experiments which had been in progress since 1893 on cider apples, peaches, pears, potatoes, melons, cabbages, &c. The liquid was held in a reservoir at a height of about 2 m. above ground-level, and was led through rubber tubing into a tapered wooden or glass tube inserted into a hole bored into the stem of the tree just above ground-level. He reported markedly good results following the injection of a liquid having the same composition as plant sap. He obtained 50 to 100 per cent. increases of crop following the injection of sea salt solution into potato plants, and an improvement in flavour and quality both in these potatoes and in similarly treated cabbages and cauliflowers.

Fron (1909), using Simon's method, injected solutions of iron sulphate and calcium nitrate into chlorotic pear trees, growing in calcareous soil. Opoix (1910) obtained good results from solid injections of iron sulphate carried out between 1905 and 1910. He reported that trees treated five years previously were still in good condition.

Italian work. Early Italian work on plant injection was restricted to attempts to control insect attack by introducing insecticides into the host. Perosino (1899) was the first to use potassium cyanide powder for this purpose. This, when injected into a bush of *Euonymus chinense* [sic], seemed to cause the scale insect *Chionaspis euonymi* infesting it to be easily blown off by the wind. According to Barbero (1899), Perosino obtained definite results of a similar kind on apple trees and on vine infested with Phylloxera. The latter apparently (Perosino, 1899) were injected in the autumn in order that the potassium cyanide might be carried 'by the descending sap' down to the roots and so to the Phylloxera parasites feeding on them. Barbero also stated that, following Perosino, similar positive results were obtained by Soave and Martinotti as well as by De Alessi and Silvestri.

About the same time Berlese (1899, 1901) injected solutions under negligible external pressure through tubing fixed directly over the ends of freshly cut root stumps, apparently in ignorance of Goff's description of the same method published in 1897, which will be referred to later. A few of Berlese's injections with tobacco extracts seem to have controlled attacks of aphides without harming the host plant; but his results were not consistent. Dezeani (1913) used injection for purely physiological purposes. He proved that potassium cyanide solution, when injected with a syringe into plants, decomposed rapidly, and he tried, but without success, to determine its fate in the plant as a means of discovering the functions of the cyanide that occurs naturally in plants.

German work. It is curious that Sach's treatment of chlorosis by injection with iron salts as early as 1880 does not seem to have been followed up to any great extent in Germany.

The work of Roth (1896) has already been mentioned (p. 160). He injected a tree with a complete nutrient solution but did not state the result. He visualized as possible advantages of the method that trees could be supplied with exactly what they needed, including their special requirements for blossom formation, and that soil deficiencies both of these nutrients and of water could be made good more economically by injection than by watering in, because when applied to the soil both are largely lost by passage to the lower layers. But, he does not appear to have followed up these ideas.

In the other German paper published during the period, Jesenko (1911) described how, by injecting 0·1 and 0·01 per cent. ether and 1 per cent. alcohol solutions into the cut ends of branches under one atmosphere pressure, he had shortened the rest period in *Robinia pseudacacia*. In Austria, in the same year, a paper was published by Weber (1911) who described experiments in which the unfolding of dormant buds was hastened by injecting water into their bases through a hollow needle.

Müller (1926) published a monograph entitled, 'Die innere Therapie der Pflanzen', in which he reviewed the literature and described his own numerous experiments. These were directed mainly to the control by injection of insect and fungus diseases of plants. He discussed at length the fact that the safety and utility of the process must depend on finding substances which are harmless to the host when injected in much larger doses than those necessary in the ordinary way to control the disease. He reported that injections of pyridine and aluminium sulphate both caused woolly aphid to leave the twigs of apple trees; but for neither was the ratio of the 'dosis tolerata' to the 'dosis curativa' sufficiently high to make the discovery of practical value.

American work. American work started quite logically with the injection of water. Goff (1897) in the spring months of 1894–5–6 injected recently transplanted trees with distilled water through rubber tubing fixed over the end of a freshly cut root stump and connected to a reservoir held slightly higher than the top of the tree. Nearly every tree so treated opened its buds,

many beginning within two days. He made the interesting observation that cut shoots injected under a $5\frac{1}{2}$ -ft. head of water absorbed one and one-half times as much water as others totally immersed until no more was absorbed.

In 1902 Bolley (1903, 1904, 1906) started work in North Dakota, a summary of which he published in 1904 and 1906, promising a bulletin which, apparently, was never published. His method was a slight improvement on that of Roth. The hole was 'at once filled with water to exclude air'. 'The economic purpose' of this work, in his own words, including italics, was '(1) if trees can be directly fed, aside from the natural source, then we can learn *what* to feed, and *how* and *when* to do it'. Thus he seems to have been the first to visualize the possibility of diagnosing a tree's nutritional requirements by injection. He proceeds, '(2) Trees are subject to two sorts of disease, (a) simple physiological derangement due to faulty nutrition, and (b) parasitic (animal and plant)'; and he hoped by injection 'to correct faulty . . . nutrition, and perhaps so medicate the food supply as to relieve or guard the plant against parasitic attacks'. In his 1906 paper Bolley stated his belief that individual unhealthy trees could be saved by injection, and he mentioned using solutions of formaldehyde ($\frac{1}{2}$ to 2 parts per thousand), copper sulphate, and ferrous sulphate, which hastened recovery of apple-trees from sun-scald and checked development of *Exoascus*. He concluded by saying: 'Plum- and apple-trees when fed have produced more and better fruit, larger growth and sturdier foliage, than checked trees. The chief difficulty in the way of this work becoming practical seems to be that each tree appears to be a law unto itself.'

Sanford (1914) stated that following the injection in February of a 12-year-old Spanish broom plant, 4 in. in trunk diameter, suffering from cottony cushion scale (*Icerya purchasi*), with enough solid potassium cyanide to fill a hole $\frac{3}{8}$ in. diameter and 3 in. deep (except for the plug), the scale was controlled in a few days and the plant became very vigorous. An old decrepit peach-tree treated in a similar manner became more vigorous and bore a crop of peaches which tests showed were not poisonous to a chicken, a rabbit, or to the experimenter himself. A year later these trees, and a similarly treated orange-tree, still showed no serious damage resulting from the treatment (Sandford, 1915). In the same year Shattuck (1915) reported that he had been using for about twelve years the injection method in his forestry work against boring and girdling insects, and it had saved the lives of thousands of trees; but he did not state how much potassium cyanide he used or the time of the year when it was injected. In the meantime, Surface (1914) reported that a commercial firm of 'tree doctors' was doing an extensive business in the Eastern States 'vaccinating' trees by inserting capsules containing potassium cyanide, potassium chlorate, and iron sulphate, into incisions made under the bark, claiming for the treatment markedly increased vigour and immunity from all parasites, both insect and fungus, and 'taking thousands of dollars from the confiding public'. Large numbers of trees throughout Pennsylvania

were killed and injured, and Surface, who examined hundreds of the treated trees, appears to have been unable to obtain evidence of any good effect from the treatment, either as regards vigour or disease. This report was followed by others by Flint (1915) and Wellhouse (1916) of injury and of ineffectiveness. After this, American work on tree injection for some years seems to have been directed along more theoretical lines. Thus Moore and Ruggles (1915) studied the movement of potassium cyanide by cutting down injected trees and applying chemical tests, and they concluded that the distribution was too localized to be of use against wood-boring insects. Elliott (1917), in more detailed work, noted that the cyanide disappeared entirely within two days and he failed to detect any movement of the salt from one annual ring to another. Two papers by Rumbold (1920*a* and *b*) contain the best review of the relevant literature up to that date.

The cure of chlorosis by injection methods, to which a large proportion of the important Russian and other work was directed, does not appear to have been attempted in America until comparatively recent years. Hendrickson (1925), as a result of his work, could not recommend injection for its control on commercial lines. Lipman and Gordon (1925), however, reported that they had cured 300 badly chlorotic trees by injecting them with ferrous sulphate solution by a modification of Roth's method. A little later Bennett (1927, 1931), after trying both liquid and powder methods for this purpose, worked out practical details for applying the latter to fruit trees of various sizes. In his 1931 paper he states that 'A total of about 75,000 pear-trees have been successfully treated by the dry-salt method, mostly by the growers themselves'. A little later Wallace (1935), in England, applied Bennett's method successfully to apples.

Allen (1931) changed the colour of Hydrangea blooms from pink to blue by injecting this shrub with solutions of aluminium salts, in this way solving a problem of some interest both from the economic and scientific points of view. He made an oblique cut in the stem upwards for some distance in such a way that the thin slip or 'tongue' thus produced could be bent aside and immersed in the solution without interrupting unduly the supply of sap from the roots. Scherer (1927) and Jacobs (1928) both reported apparent cures of fungus disease without damage to the host following the injection with thymol in solution.

During the last decade, injection methods have been increasingly used for the study of mineral nutrition in fruit trees. Lipman and Gordon (1925) published the results of injecting twenty-four 10-year-old pear-trees with eleven nutrient solutions. The most striking effect was that produced by magnesium, whether used as nitrate or as monohydrogen phosphate. The foliage of four trees injected with these salts assumed a deep green colour a few weeks after treatment. Collison, Harlan, and Sweeney (1932) published a paper entitled, 'Direct tree injection in the study of tree nutrition problems', in which, as the title suggests, attention was concentrated on the value of

the method for research purposes in tree physiology and nutrition. They pointed out, independently of the present writer (Roach, 1931, see later), that the soil often reacts chemically with substances applied to it, and these may never reach the roots of the trees, or become changed during passage through the soil before reaching them. Injection methods hold out the possibility of avoiding these disturbing effects of the soil and consequently of testing the direct effects of the actual substances on the tree. These workers attempted to inject individual branches of a tree, using both Roth's and a branch-stump method (see p. 198). They analysed parts of the trees to test the effect of the substances injected on the chemical composition of the tree, and they pointed out how natural variation in such composition between one branch and another of the same tree complicates experimental work on injection.

Chandler, Hoagland, and Hibbard (1933) cured 'little-leaf' or 'rosette' of fruit trees by injecting them with zinc sulphate, and Demaree, Fowler, and Crane (1934) cured pecan 'rosette' in a similar manner.

English work. Brooks and his co-workers used injection methods both in the first stage of an attempt to cure plum-trees suffering from silver-leaf disease (*Stereum purpureum*), and for a purely physiological purpose. He and Bailey (1919) injected plum-trees by Goff's method with solutions of dyes and disinfectants, and the larger number of recoveries amongst the injected trees as compared with untreated controls suggested to them that the fungus had been killed by some of the substances injected. Brooks and Storey (1923) determined the toxicities of a number of fungicides towards the fungus grown on an artificial medium. The most toxic of these, 8-hydroxyquinoline potassium sulphate (known also as ortho-oxyquinoline potassium sulphate, and commercially as superol or chinosol), has been used successfully by Wormald and the writer in preliminary unpublished experiments on the control of this disease in plums. Quite recently Fron (1937 and 1937a), whose earlier work has already been noted, induced a darker green foliage colour and an increased vigour and resistance to diseases in carnations to *Fusarium Dianthi* and in elms to *Ceratostomella Ulmi* by injecting the attacked hosts with 0·05 per cent. solution of the neutral sulphate of the same organic compound, known commercially as cryptonol or sunsol. His work is being applied successfully on a commercial scale for carnations; the results so far obtained with elms encourage the hope that this tree may be saved from threatened extinction by the disease.

Reverting to the investigations of Brooks and his co-workers, the injection method was used by him and Moore (1926) and later by him and Brenchley (1929, 1931) to prove that aqueous extracts prepared from cultures of *Stereum purpureum* on artificial media when injected into plum-trees produced silvering of the foliage and other symptoms of the disease; and they determined some of the properties of the substance or substances causing these symptoms.

The present writer has been working on plant injection for some years and

published preliminary notes on his results in 1931 and 1933 and also a paper in 1934 entitled 'Injection for the diagnosis and cure of physiological disorders of fruit trees' (Roach, 1934). More than half of this paper was devoted to the principles underlying the control of the distribution of injected liquids. It was necessary fully to understand these before it was found possible to cause at will the uniform permeation of all the branches when a whole tree is injected, or to prevent the liquid from travelling beyond the branch or shoot concerned when only a part of a tree is to be injected for diagnostic purposes. These and other preliminary notes and papers will be referred to in the next part of this paper.

Injection work on mineral deficiency diseases.

Of late years various injection methods have been used as a first, or an early, step in finding cures for a number of deficiency diseases, and the investigations concerned with this have been carried out along essentially similar lines in practically all countries. The American work on zinc has already been mentioned (p. 165). Anderssen (1932), in South Africa, cured experimentally on a few leaves a chlorosis of deciduous fruit trees by injecting the leaves with copper sulphate solution. His method is an interesting one: 'Test tubes filled with a solution of CuSO_4 (0·3 p.p.m. Cu) were fixed to trees with strips of adhesive tape and a chlorotic leaf was bent so as to dip into the solution in each tube. . . . After two weeks the particular leaves which had been immersed in the CuSO_4 solution had turned perfectly green whereas the rest of the leaves on the same twig were still chlorotic. . . .' This point is vividly demonstrated by his plate. Further, he states that 'Occasionally the whole twig may turn green'.

Storey and Leach (1933) in East Africa cured experimentally a chlorosis of the tea-bush by injecting it with solutions of sulphates. Leach's method (Storey, 1938), which will be mentioned again later (see p. 198), resembled that of Collison, Harlan, and Sweeney (1932, see p. 198) for branches, but was on a smaller scale. Atkinson (1935), in New Zealand, McLarty (1936) and Young and Bailey (1936) in Canada, Jamalainen (1936) in Finland, cured 'cork' troubles in apples by injecting trees with boron compounds. The practical value of some of these discoveries will be discussed later.

Injection methods used in physiological work.

Finally, injection methods have been used with success for purely physiological purposes. Moreau and Vinet (1932) more than doubled the number of grapes set by injecting glucose into a vine just prior to bud-break. Their method was a new one. They wrapped the glucose in filter paper and placed it in a test-tube, the end of which was drawn out and pushed firmly into a cork. This was pressed into a hole bored into thick healthy tissue in the trunk or branch, the joint being made water-tight with paraffin wax. The sap rose into the tube and dissolved the contents.

Iyer, Siddappa, and Subrahmanyam (1934) injected plants with certain solutions of organic nature by means of a hypodermic needle attached to a reservoir containing the liquid, and they observed marked effects on growth and sexual reproduction.

Oinoue (1935), by injecting both glucose and asparagin into grape vines by Rassiguier's method, varied both the total amounts of carbohydrate and nitrogen and the carbon to nitrogen ratio. In consequence he was able to study the effect of all three of these factors on the setting of the fruit. Other examples will be given in the next part of this paper.

The purpose of the foregoing outline of the history of plant injection has been to show the general trend of past work; a more complete review is being published elsewhere (Roach, 1938). A number of papers published while the present investigations were being carried out will be considered further in the final discussion, because they illustrate how the methods now to be described may be applied to practical problems.

Scope of Present Paper

The remainder of this paper will be devoted to an account of the methods developed for injecting, in standing trees and plants, particular parts varying in size from a single interveinal area of a leaf to a whole main branch, each with a different liquid; the method for injecting whole trees will also be described. Brief mention will be made also of the types of problem for the solution of which each method of injection is best suited. The most delicate methods, in which parts of leaves are injected and compared with contiguous untreated or differently treated parts of the same leaf, make possible the diagnosis in a few days of mineral deficiency, when the treatment results in a change in leaf colour or growth rate. When the effects on vegetative growth, development of disease, or crop are to be studied, the injection of a number of separate branches of suitable size on a single tree often enables comparisons to be made conveniently and accurately. Whole trees may be injected for experimental purposes, and in special circumstances their injection on a commercial scale is warranted.

For these purposes an accurate knowledge of the distribution of the substances after injection is essential. For example, in injection for diagnostic purposes, when a number of branches or twigs or smaller parts of the same tree are treated each with a different liquid, it is necessary to know on the one hand how much of each branch or leaf will become completely permeated, and on the other hand the point beyond which none of the liquid will pass. When whole trees are injected it is desirable that all branches should become uniformly permeated.

Material

The experiments have been in progress during the last seven years and the material used has varied from strawberry plants and nursery stock to fully grown plum, apple, and other trees. A whole plantation of partially

derelict plum-trees, which was felled while the experiments were in progress, provided valuable material not often available for such purposes; and a number of healthy, nearly full-grown apple-trees which also became available were of even greater value. The work has hitherto been confined mainly to trees, bushes, and plants cultivated for fruit production, and little attention has yet been paid to the attractive problems likely to arise when the methods are extended to other plants, differing in leaf venation, branching, and other morphological characters.

METHODS OF INJECTION

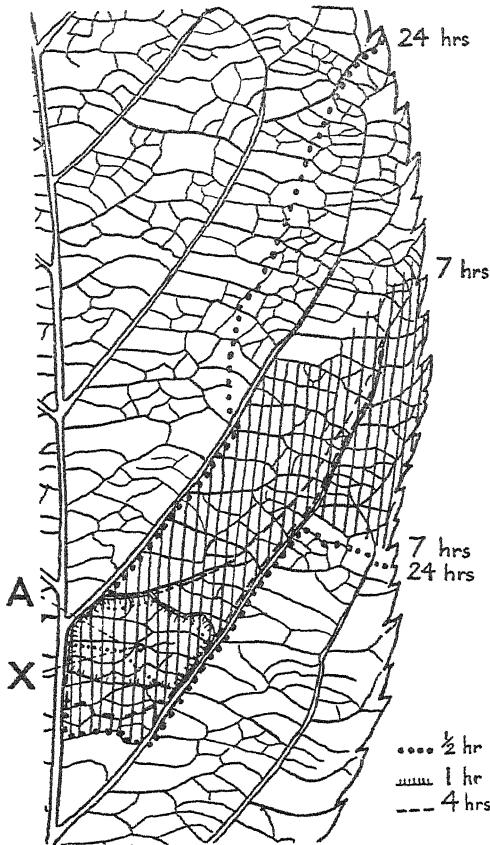
Many different methods of carrying out injections are possible, and they are most conveniently discussed and explained in relation to the size of the part of the plant to be treated. All methods may be employed for diagnostic purposes, each having its own range of usefulness; but only those affecting the whole tree, and to a less extent those designed for treating main branches, are likely to be of use for commercial purposes. The chief methods of injection evolved and employed up to the present will now be described, beginning with those for use on a small scale and most suitable for rapid diagnosis, and concluding with those which affect the whole plants. Full practical details of methods now to be briefly described are given in a paper elsewhere (Roach, 1938).

I. *Interveinal Leaf Injection*

A preliminary experiment.

As is well known, the loss of water from the leaf surface by transpiration brings the water in the conducting system into a state of tension, and if these tissues are opened at any point under water the water is drawn in. This fact is readily demonstrated by pushing the wet capillary tip of a glass tube containing a suitable dye in aqueous solution just barely through the blade of a leaf still on the tree. In this way the ruptured cells immediately become connected by a film of liquid with the dye solution in the tube, and the movement of the dye may be watched. Its progress and the final distribution of the dye vary according to the venation of the leaf and the position of the rupture. The results of such an experiment on an apple leaf, with reticulate venation, are shown in Text-fig. 1. The point of insertion of the capillary tube, shown at *x*, was equidistant from two neighbouring secondary veins and from the midrib, and a veinlet *a*, connecting the midrib with a secondary vein was perforated. The leaf and injection tube remained in contact at this point and the dye solution (0.5 per cent. patent blue) travelled in both directions along this veinlet until it reached the midrib and the lower of the two secondary veins. At the same time it spread more slowly upwards and downwards in the tissues adjacent to the veinlet, but did not cross the midrib or either of the secondary veins. Half an hour after the puncture was made the limits of spread of the dye were as indicated by the light dotted line (see key on Text-figure 1), and about five minutes later it had reached the limits which

in the figure mark one hour's spread and are shown thus ---- . Although the colour of the area permeated deepened, the dye did not progress farther upwards for more than half an hour. It was 'held up' all this time along the



TEXT-FIG. I. The numbered lines mark the limits of permeation, after varying times, of a dye solution injected through an incision X in an apple leaf. The veinlet marked A was punctured by the incision.

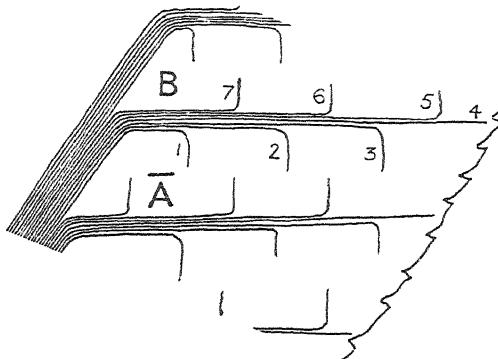
line of another veinlet stretching from one of the secondary veins to the other, but at the end of this time, i.e. one hour after the start, the dye began to travel along the lower secondary vein, and later it spread slowly along it towards the midrib. The roughly triangular area enclosed by the interrupted line (see key) was that occupied by the dye four hours after the start. Comparatively little movement had taken place in the direction of the midrib and none across it or across the two secondary veins. The dye had still not crossed the whole of the veinlet just referred to. Seven hours after the start (see hatched area)

the dye had crossed the upper end of the lower of the two secondary veins and had spread to the margin of the leaf; it had also spread along more than half the length of the upper of the two secondary veins, but had not crossed it or the midrib. Even after twenty-four hours (see heavy dotted line) the movement of the dye was limited by the midrib and the two secondary veins, except at the upper ends of the latter where the dye had spread across them. The much slower progress of the dye downwards may be followed in the figure. Most of the above facts are in harmony with what is already well known. The dye solution was sucked in under the liquid tension existing in the vascular system of the leaf due to the transpiration of water from the leaf surface. The solution travelled comparatively freely along the elongated elements of the veinlets, its flow along them towards the leaf margin being limited by the high resistance offered by the large surface area of the fine vessels through which it travelled and also by the constant loss of water by evaporation at the leaf surface. The flow towards the main vein was further limited by the normal flow of fluid in the opposite direction, and the dye solution, therefore, spread farther towards the leaf margin than towards the main vein. Its more rapid movement along than across the veinlets is explained by their structure. Each consists of a bundle of elongated tubes lying side by side, the tubes being divided at intervals by cross walls or septa pierced by pores through which liquid may pass. These tubes have also a smaller number of pits in their side walls, through which liquid can pass from one tube to another, but movement from tube to tube laterally is not nearly so easy as that along the length of the tubes. Further, sap was already being drawn along them before the injection started, consequently the injected liquid, in order to travel laterally, would have to move across, not stationary canals, but rapidly flowing streams. In a secondary vein the tubes in continuation of many veinlets lie side by side, and consequently offer a still greater resistance to flow across it; in fact, the larger secondary veins and the midrib of the apple leaf are hardly ever crossed by liquids injected as in the above experiment, except at their upper ends where they become thin.

The way in which the injected liquid permeates the whole of the interveinal area between the injection point and the leaf margin before travelling into the neighbouring interveinal areas would hardly be predicted on the basis of our knowledge of the manner in which the veinlets are collected and to some extent fused to form a secondary vein, which in turn joins with the midrib. The facts are illustrated diagrammatically in Text-fig. 2, in which each of the lines labelled 1 to 7 represents a veinlet and the conducting tissues in continuation of it in the secondary vein and midrib. For convenience these will be called *strands*. Throughout this paper, for want of a better term, this word will be used to denote long thin pieces of conducting tissue. Only three veinlets are represented on each side of each secondary vein in the figure, whereas there are more than ten times that number in the actual leaf. There are therefore more than seventy strands separating the injection point A from

a point B in the next interveinal area. As the dye enters each strand it travels slowly backwards for a short distance and rapidly upwards to the end of the strand. Its direction and the successive positions reached by it are therefore represented by the order of numbering of the strands. Strand 1 becomes permeated for its whole length and later strand 2 is permeated in a similar manner. The dye may travel, apparently, around the end of the secondary vein, but actually it will permeate strands 3, 4, 5, successively.

The secondary veins are represented in the figure as flat sheets of strands.



TEXT-FIG. 2. Diagrammatic representation of the arrangement of the vascular system in direct connexion with the veinlets, which are numbered 1-7, as deduced from the results of injections through an incision such as at A. The numbered pieces of vascular tissue are, for convenience, called strands in the text.

Actually they are roughly circular in cross-section, and the strands, therefore, are closer to each other than shown in the figure; in fact they are contiguous or fused. The above facts prove that the strands are 'packed' in the veins in such a way that liquid can move from one to another only in the order mentioned, and not, for instance, direct from strand 1 to strand 7, the corresponding strand on the other side of the vein. These facts suggest that the strands are flattened into thin strips in parallel planes at right angles to the surface of the leaf blade.

Position of injection point.

As will be described presently the injection is usually through a small incision in the leaf blade. Such an incision interferes least with the normal flow of sap when it is made midway between two neighbouring, substantial veins, since it does no damage to any main or secondary vein or even to any large veinlet. If a main or secondary vein is damaged, all the tissue traversed by veinlets joining it nearer the margin may die. Wherever the incision is made the injected liquid will reach the leaf margin within about five hours, but it travels only a comparatively short distance towards the midrib.

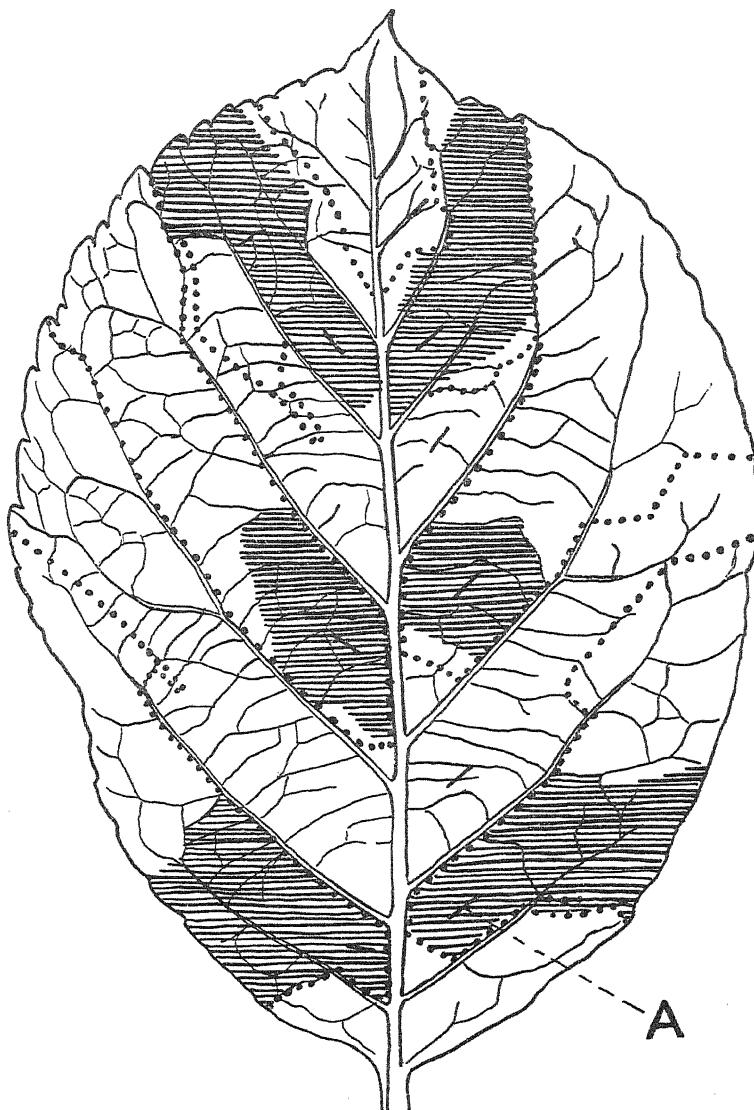
Therefore only when the incision is made in the position selected in the experiment just described, or a little closer to the midrib, does the tissue close to the midrib become permeated. Further, the closer the incision is to the midrib the less is the risk of the liquid crossing a secondary vein into the next interveinal area, since the secondary veins increase in thickness as they approach the main vein. This shows that the best position for the incision is the one selected.

Distribution of injected substance.

Text-fig. 3 shows the distribution of a solution of patent blue injected through a number of incisions (one is marked A) made at such points in alternate interveinal areas on both sides of the midrib. The areas permeated at the end of four hours are cross-hatched, and the edges of those invaded in the following nineteen hours are shown by dotted lines. The remaining interveinal areas on the left-hand side of the leaf were untreated, but those on the right-hand side were injected with water at the same time as the dye injections were started; this had, however, no appreciable effect on the distribution of the dye. Such injections, therefore, may be made in the apple leaf on the assumption that well-developed secondary veins are practically impassable barriers for liquids introduced at a point from the midrib not more than half-way to the leaf margin, whether the neighbouring interveinal areas are injected or not. Even when the secondary veins become crossed there is never any uncertainty as to which injection was responsible for the resulting colour or other change, since the injected area is always continuous and never subdivided, and always has within it the incision through which the injection was made. The pair of areas lying between the base of the midrib and the first secondary vein on each side of it, as well as the terminal quarter of the leaf, are less suitable for interveinal injection than the remainder of the leaf for the reason that when the incision is made near either the tip or the base of the leaf the permeated area is less clearly demarcated than when it is made near the middle.

Duration of injection.

The above and similar experiments have proved that unless injection continues for some time the permeated area does not extend far alongside the secondary veins. The effect of the injection can be observed best when permeated and untreated areas are sharply separated by a continuous boundary of as great a length as possible. These conditions were satisfied in the preliminary experiment described when the injection had proceeded for seven hours; the area then invaded is hatched vertically in Text-fig. 1. At this stage the secondary vein on each side of the incision formed a sharp boundary for rather more than half its length between permeated and normal areas. Text-fig. 1, and even more so Text-fig. 3, shows that if the injection is allowed to proceed too long (about twenty-four hours), the injected liquid tends to



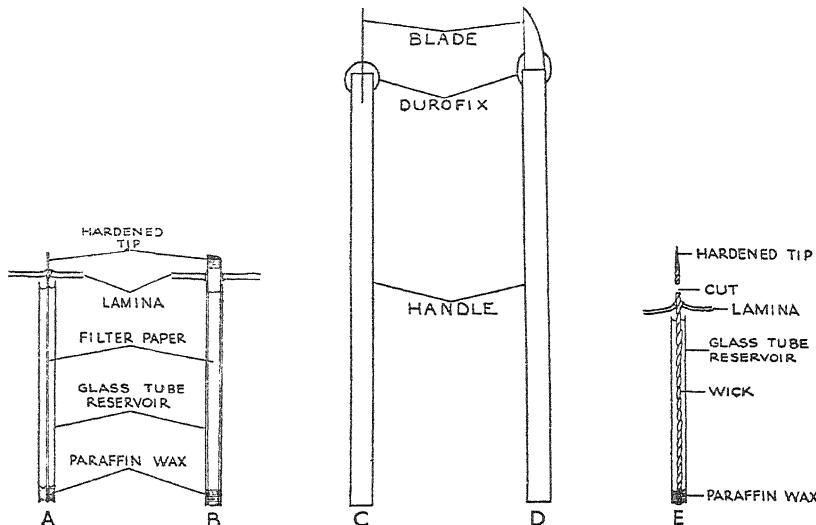
TEXT-FIG. 3. Result of interveinal injection of an apple leaf. The cross-hatched areas were permeated in 4 hours by a solution of patent blue injected through a number of incisions, one of which is marked A. The limits of permeation after 23 hours are marked by dotted lines. The alternate interveinal areas of the right half of the leaf were injected with water but it had no effect on the distribution of the dye.

creep beyond the secondary veins, and the divisions between permeated and uninjected areas cease to be so definite as when the secondary veins themselves constitute the boundaries. The optimum duration of injection varies

somewhat from one type of leaf to another and according to the meteorological conditions; consequently, to obtain the sharpest contrasts, preliminary experiments should be carried out with dyes. With leaves of apple, pear, strawberry, and Shasta daisy, however, a duration of from seven to twelve hours has been found satisfactory.

Methods suitable for use in field.

The glass tube drawn out to a capillary tip used in the preliminary experiment is quite convenient for single injections in the laboratory, but is useless



TEXT-FIG. 4. Apparatus for interveinal leaf injection. A and B are two views of the same apparatus, B being at right angles to A. C and D show in two views the scalpel for making the incision for interveinal injection. The view D is at right angles to C. E, apparatus for interveinal injection of small leaves.

for experiments in the open or when several injections have to be done on a single leaf. Two more convenient types of apparatus are illustrated in Text-fig. 4.

Method I. The first method is illustrated diagrammatically in Text-fig. 4 A, B. The liquid, held in a reservoir, is drawn up a filter-paper 'wick' which passes through an incision made in the leaf. The 'lips' of the incision clip the wick, which in turn supports the reservoir. A film of liquid connects the cut tissues with the wet filter-paper. The tissues absorb liquid from the film and liquid travels up the filter-paper to make good the loss.

Method II. The second method is simpler than the first and is applicable to smaller leaves. In it the filter-paper 'wick' is replaced by a piece of darning cotton which serves the same purpose. The method is illustrated in Text-fig. 4 E.

Time of detection of response to injection.

As already pointed out, the conditions under which the treated and untreated areas of leaf are compared are nearly ideal, in that they are separated

only by a narrow secondary vein and in that the comparison can be made along both sides of the treated area. In these circumstances a slight colour change is readily detected which would be quite impossible by other methods, as for example, when comparison is made between whole plants, the individual leaves of which usually show considerable variation in colour. The quickest response so far observed was given by an injection, made recently by Lal, of 0.025 per cent. ferrous sulphate into a soya bean leaf, there being a definite intensification of green two days later; this work will be published in due course. In an earlier experiment (Roach, 1937a) an injection of 0.05 per cent. iron citrate solution into a chlorotic apple leaf (see p. 176), led three days later to a slight but quite definite green tint. The green colour increased in intensity for a further seven days. In other experiments, seven days have elapsed before a response has become definitely discernible. Experience so far obtained suggests that the full response is given in ten days.

Effect of age of leaf on response to injection.

The experience obtained with this second method, which so far is somewhat limited, suggests that a half-grown leaf is the best. Responses have been obtained with immature leaves when mature ones have given no visible response. Further, when such immature leaves have been injected there has sometimes been observed, in addition to an improvement in colour, greater growth of the permeated as compared with the untreated areas; this naturally has resulted in a puckering of the leaf.

Substances injected.

Because of the striking colour change brought about by the injection of iron compounds into one type of chlorotic leaf most attention has so far been paid to this element, but experiments are being carried out with others including the principal nutrient ones.

Hill, in work to be published in detail shortly, has diagnosed by these methods or slight modifications of them (see below) nitrogen deficiency in broad bean, and phosphorus and boron deficiency in tomato plants.

Pl. IV, Fig. 1, reproduced by the kindness of Mr. Hill, shows the results of injecting an interveinal area of a broad bean leaflet with urea. The effect was only just detectable with certainty by the naked eye, but photography made the effect much more apparent.

Kinds of leaves.

The methods described have been applied successfully to apple, pear, and plum leaves, and strawberry leaflets, the venation of all of which is reticulate-pinnate. Hill, as stated above, has used them successfully on the broad bean and on mature tomato leaflets; but when applied to young tomato leaflets (Pl. IV, Fig. 2) the liquid does not remain confined within the area delimited by the two secondary veins on each side of the incision. The writer has had

the same experience with peach leaves. Presumably the number of veinlets united in the short secondary veins of these long narrow leaflets or leaves is insufficient to present a serious barrier to the movement of injected liquid across them. The conditions, in fact, are similar to those in the apple leaf in the case where the incision is made midway between the main vein and the leaf margin or even nearer the leaf margin, when, as has been shown, the liquid crosses the secondary veins. In working with young leaflets, such as those of the tomato, a half leaflet is the smallest practicable injection unit. The incision is made near the base of the leaflet on one side of the midrib. The injected liquid then remains on one side of the midrib and the tissues on the other side of it may be used for comparison. In the peach a half-leaf is the smallest practicable unit.

Application of interveinal method.

The problem for which the method was actually evolved will serve as an example of its practical application (Roach, 1937a). Small apple trees grown in a nutrient solution developed severe chlorosis. In the absence of any definite indication as to which element was lacking the effects of a number of nutrient salts, both alone and in combination, were tried by this method. In three days one interveinal area injected with iron salt was slightly greener than the surrounding tissues, and on the fourth day this and two other areas injected with an iron salt combined with other nutrient salts were definitely darker green than untreated areas, thus proving in four days that the trouble was due to iron deficiency. A week after the injection the permeated areas had assumed a healthy glossy dark green colour, and the fact that these areas were raised in puckers demonstrated that they had grown faster than the surrounding untreated tissues.

Because the treated areas were so small these tests caused the small trees no appreciable damage. For the same reason it was possible to prove that the gradation in colour shown by the leaves on each shoot of these trees was due to an iron deficiency which became more severe as each succeeding leaf unfolded, and, a point of greater importance, the early stages of iron deficiency were demonstrated. Each of the first three or four leaves was of a lighter green than its older neighbour, the green was of a 'metallic' quality, and the leaves appeared to lack 'finish'; the leaves succeeding these showed the well-known symptoms of iron deficiency chlorosis, namely a yellowing around the leaf margin, especially toward the tip, tending to spread between the veins towards the midrib in severe cases. In very severe cases even the veins themselves were of a yellow colour and quite free from green. The early stages of this condition, characterized merely by a light unhealthy type of green, had not previously been associated with iron deficiency. The fact was proved by injecting leaves in all stages and obtaining an improved green colour except in the darkest green ones; and the importance of even this early stage of the trouble was suggested by the increased growth of the injected

areas. This condition of iron deficiencies appears to be widespread throughout Kent and elsewhere (Roach and Levy, 1937).

Statistical significance of results of interveinal leaf method.

This must obviously depend on how often there occur in nature single interveinal areas, of healthier appearance than those areas on each side of them such as could be mistaken for the effects of artificial interveinal injection. Careful search has been made for the past two seasons, but among the many thousands of leaves examined in orchard and garden only a single such instance has been observed. The odds against the selection of that particular area for an injection are therefore more than 1,000 to 1; and if the experiment is done in duplicate the odds become 1,000,000 to 1. This is a degree of certainty rarely achieved in biological experiments. Moreover, the above calculation takes no account of the fact that an actual change is observed (e.g. of colour), and that this takes place during a predictable and relatively short period of time. This fact still further enhances the trustworthiness of the method.

The above considerations apply whenever a positive result is obtained by this method, no matter how small the change in the appearance of the area may be. But when, for example, the colour of the area changes as a result of an injection from yellow to a much darker green than that of any leaf on the plant the diagnosis approaches complete certainty.

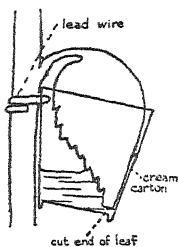
II. Leaf-tip Injection

Simple leaves.

This method consists in cutting off the tip of a leaf or a leaflet at right angles to the midrib and at once submerging the cut edge below the liquid to be injected. The method is particularly suited to long narrow leaves such as those of the peach, but it may be used for almost any type. A convenient mode of procedure is illustrated in Text-fig. 5. The liquid is drawn in through the cut edge of the leaf under the tension set up by transpiration, and as it travels along the veins and veinlets it is continually used by the leaf tissues to make good transpiration losses. Its flow is of course impeded by the high frictional resistance offered by the large surface of the fine spaces through which it flows; the extent of the injection is also limited by the flow of sap in the opposite direction.

The greater the distance of the cut from the leaf tip, the larger is the number of veins and veinlets severed, and consequently the greater is the distance from the cut edge that may be reached by the liquid; this statement will have to be modified when leaves with parallel venation are injected. It is necessary to make preliminary injections with dye solutions with each kind of leaf to decide how much of the tip to remove to obtain any desired degree of permeation, but the following figures will give some idea of results likely to be obtained with apple or pear leaves. If a portion equal in length to one-tenth

that of the midrib is removed about half the leaf will become permeated; if equal to one-fifth the whole remaining part of the leaf will become permeated; if equal to more than one-fifth not only the rest of the cut leaf itself but part or all the basal halves of the two leaves, situated respectively above and below the treated one on the twig, will also become permeated. It is best, therefore, to aim at permeating no more than three-quarters of the leaf, so that there shall be no risk of the injected liquid travelling into other leaves, which would limit the choice of an untreated leaf, as a control. A consideration of the results of leaf-stalk (petiole) injection in the next section will make this matter clearer.



TEXT-FIG. 5.
Leaf tip injection. A cream carton is held by 'lead' wire to the stem and the leaf bent down into the solution it holds.

With apple and pear leaves enough liquid is usually absorbed in about ten hours, and the response to the injection of an iron salt by this method is usually apparent in a week or ten days (Roach, 1936). The slight increase in greenness, which is detectable within three days by the interveinal method of injection previously described when comparing areas on each side of a secondary vein or the midrib, is usually quite imperceptible when comparing an injected whole (tipless) leaf with an untreated control. Hence although the leaf tip method is easier to carry out the interveinal method is preferable when rapidity of response is essential. Nevertheless, the leaf tip method may have advantages for certain physiological experiments.

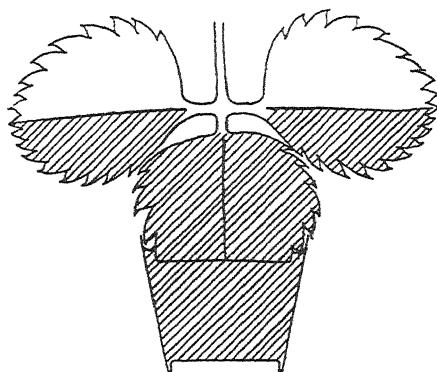
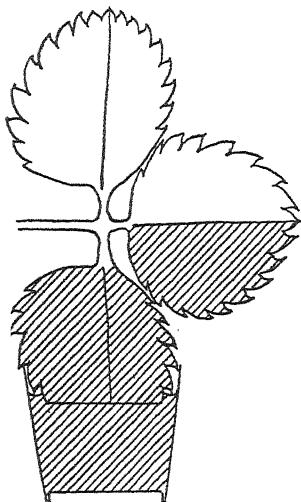
Compound leaves.

(i) Each leaflet of a compound leaf may be injected separately by cutting off its tip and immersing the cut edge of the leaflet in the liquid to be injected. Thus, one lateral leaflet of a strawberry leaf may be so treated and the other left untouched for comparison. (ii) If the cut is made so as to remove one-quarter or a little more of a strawberry leaflet the injected liquid travels into the nearer side of the adjoining one. Thus, after a little practice with dyes, it is possible to inject through the cut tip of a lateral strawberry leaflet in such a way as to affect the whole of that leaflet and almost exactly half of the terminal one (Text-fig. 6) in which the treated and untreated tissues will be sharply separated by the main vein for most of its length. If the terminal leaflet is injected in a similar way, it and the nearer half of both the lateral leaflets are permeated (Text-fig. 7).

Statistical significance of results of leaf-tip method.

The degree of certainty with which a symptom of improved health in an injected leaf can be attributed to the injection must depend on the proportion which the number of leaves of equally healthy appearance bears to the total number of comparable untreated leaves on the tree. If the appearance or

size of the injected leaf is not beyond the normal range of variation of the leaves of the tree the trustworthiness of a diagnosis can be calculated quite simply by the ordinary laws of chance; but should the appearance or size of the leaf definitely exceed this range of variation the certainty of the diagnosis is greatly enhanced. An example will make this point clear: If the leaf after injection becomes the most healthy in appearance of, say, the 100 leaves on



TEXT-FIGS. 6 and 7. Fig. 6. Injection of strawberry leaf through the tip of a lateral leaflet. Fig. 7. Injection of a strawberry leaf through the tip of the terminal leaflet. Cross-hatched areas are permeated.

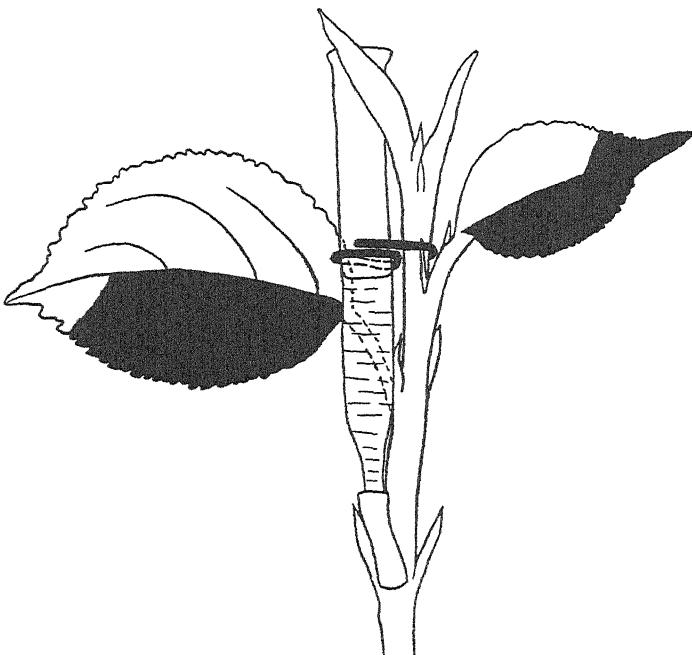
tree it may be argued that the odds against this one leaf being by chance the healthiest are 100 to 1; and this statement would give a measure of certainty of the improved health being the result of the injection. When, however, the appearance of the injected leaf is definitely outside the range of variation of the leaves on a tree, e.g. when all the leaves on a tree are chlorotic and the injected one takes on a healthy green colour after being injected with a solution of an iron salt (Roach, 1936), the change in condition is practically certain to be due to the substance injected and the reliability of the diagnosis is almost absolute.

III. Anderssen's Leaf-immersion Method

Anderssen's own description of this interesting method has been given earlier in this paper (p. 166), but the writer has so far had practically no experience of it. A comparison of the results given by it and by the preceding method would be of obvious interest, and this will be made as soon as opportunity offers.

(a) *Apple.*IV. *Leaf-stalk Injection*

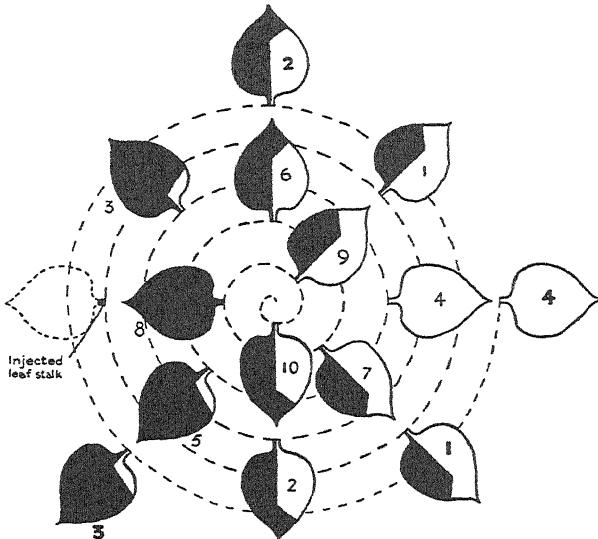
The fact already mentioned that when more than one-fifth of the leaf is removed in preparation for leaf injection the injected liquid travels into other leaves suggested that useful types of distribution of the injected liquid might be obtained by removing still more of the leaf. Descriptions of a few experiments in which the whole of the blade was removed and the leaf-stalk remaining was injected with dyes will indicate some of the kinds of distribution obtainable.



TEXT-FIG. 8. Leaf-stalk injection. The liquid is held in a glass tube which is attached to the leaf-stalk stump with rubber tubing. The permeated areas are shown black.

A leaf towards the base of a current year's apple shoot was cut off so as to leave a short length of stalk attached to the twig. To this was connected the drawn-out end of a small test-tube by means of bicycle valve tubing, the upper end of the tube being fixed to the stem with lead wire or rubber surgical tape (Text-fig. 8). The tube was filled by a hypodermic syringe with 0.5 per cent. aqueous solution of acid fuchsin, care being taken to wet the cut end of the leaf-stalk and to expel all air from the narrow rubber tubing. In a few minutes there was a perceptible reddening of parts of certain of the neighbouring leaves, and the distribution of the dye could be mapped after about fifteen minutes. The intensity of this reddening continued to increase but there was no further spread of the dye at the end of twenty-four hours.

The resulting distribution of the dye is shown diagrammatically in Text-fig. 9, which is drawn as if the leaves had been detached from the shoot and



TEXT-FIG. 9. Phyllotaxis and leaf-stalk injection pattern of the apple shoot. The injected leaf-stalk is seen on the left. Permeated areas are shown black.

arranged at points on a phyllotaxis diagram corresponding with their positions on the stem. The results, expressed in a different form, are also shown in Table I.

TABLE I

Leaf.	Fraction of leaf blade permeated.	'Angular' distance from injected leaf-stalk.
Above injected leaf-stalk	10 Slightly more than half.	2/8
	9 Slightly less than half.	3/8
	8 Whole.	0
	7 Slightly less than half.	3/8
	6 Slightly more than half.	2/8
	5 Nearly whole.	1/8
	4 None.	4/8
	3 Nearly whole.	1/8
	2 Slightly more than half.	2/8
	1 Slightly less than half.	3/8
Injected leaf-stalk L		
Below injected leaf-stalk	1 Slightly less than half.	3/8
	2 Slightly more than half.	2/8
	3 Nearly whole.	1/8
	4 None.	4/8
	5 Nearly whole.	1/8

The fraction of the area of the leaf permeated by the dye decreased as its angular distance from the injected leaf-stalk (L) increased; the leaf directly 'above' it (no. 8) was permeated throughout its whole area, whereas the fourth leaf up the spiral, which is on exactly the opposite side of the stem to the injected leaf-stalk, was not reached by the dye. The corresponding leaf (4) down the spiral was also quite free of dye. Leaves 3, (3), and 5 (i.e. the third leaf up, the third leaf down, and the fifth leaf up the spiral), which were distant only $1/8$ circumference from the injected leaf-stalk, were permeated except for a small area at the base of the blade on the side remote from the injection point. Leaves, 2, 10, 6, (2) (distant $2/8$) were coloured over slightly more than half their area; and leaves 1, 9, 7, (1) (distant $3/8$) were permeated over slightly less than half their area. The diagram makes clear the fact that the permeated part of a leaf extended as an undivided area from the base of the blade on the side nearer to the injection point, up that side of the midrib, nearly to the leaf tip; in fact, no other part of the leaf became permeated unless this proximal basal area was permeated. Other experiments showed that the actual fraction of the leaf areas permeated could be decreased below that shown in Text-fig. 9 by making the cut nearer the leaf tip. As already stated (p. 177) if the length of the part cut off at the tip is not more than one-fifth that of the midrib no injected liquid reaches other leaves. Hence, as the length of tip removed increases from one-fifth to the whole blade together with most of the leaf-stalk, so the permeated areas of other leaves on the shoot increase from zero up to those shown in Text-fig. 9. As the amount removed from the injected leaf increases so, for example in leaf 3, the permeated area increases. At first a small basal area on the proximal side of the midrib is affected, then this increases up its proximal side, rounds the tip of the leaf and extends down the distal side until only a small area, such as that shown in the figure is left unaffected.

In eight experiments (four with 0.5 per cent. acid fuchsin and four with 0.5 per cent. patent blue) in which the cut was made half-way along the leaf-stalk of a leaf near the middle of the current year's shoot, the resulting distributions were practically the same. In these experiments the liquid travelled rather less freely downwards than upwards, reaching the tenth leaf upwards but only the fifth downwards. In other experiments, in which leaf-stalks nearer the top of the shoot were selected, so that the upward movement was limited by the number of leaves above the injected leaf-stalk, the downward movement tended to increase rather than decrease; and when the stalks of leaves which had just become fully expanded were used movement downwards was actually greater than that upwards. Closely similar results were obtained with shoots which had finished extension growth. The distribution of dye in leaves which were still folded followed the same rules.

These facts show that liquids may be injected into apple leaf-stalks by this method with considerable confidence as to the actual type of distribution attained, and experiments with colourless liquids can be designed so that the

most sensitive leaves, i.e. the youngest, become injected in such a way as to be of the most use for diagnosis. Perhaps the most generally useful arrangement is to inject the stalk of the youngest leaf that is stiff enough to hold the rubber tubing. The two or three leaves above it are usually still unfolding, and consequently are likely to respond fully to any nutrient supplied; moreover there are also two or three leaves below, any of which may not be too old to show some degree of response. In all of these leaves the basal proximal quarter becomes injected and the corresponding distal quarters remain uninjected. In the basal halves of these leaves, the treated and untreated areas are divided sharply from each other by the midrib vein; this is an almost ideal arrangement for showing any small difference in colour, rate of growth, &c., and which has proved of great use in diagnosing iron deficiency.

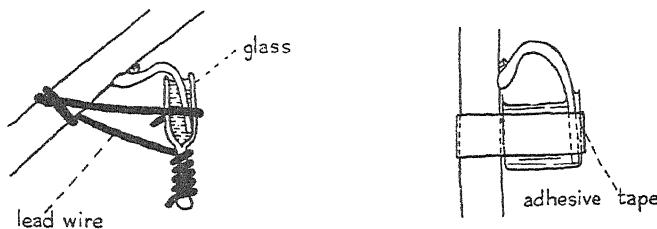
Sixteen injections of 0·05 per cent. solutions of iron citrate and ferrous sulphate have been carried out on apple trees in the Research Station plantations (Roach and Levy, 1937). The distribution of a 'bronzing' effect, which resulted in a few injections from slightly excessive doses, and the increased depth of green observed later in these experiments, followed the same laws as those governing the distribution of dyes.

Sectorial injection of apple fruit. Preliminary experiments carried out by Mr. Levy with dyes have shown that if a leaf-stalk injection be carried out on a spur carrying a fruit either the whole fruit or only a single sector of it may be permeated, according to the position of the injected leaf-stalk in regard to the fruit. The size of the permeated sector may be varied in a manner similar to that in which the area of permeation of leaves may be controlled.

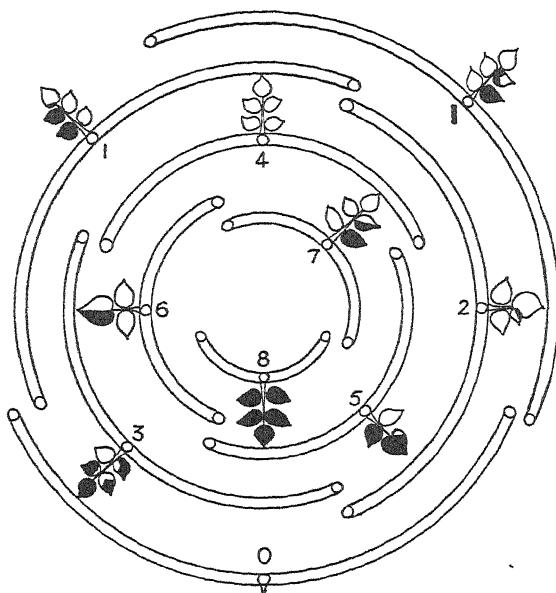
Application of leaf-stalk method. The comparison of treated and untreated areas is nearly as sharp in this method as in the interveinal one, consequently any response to the injected substance can be detected nearly as surely and quickly. It is easier to carry out and is less liable to be vitiated by rain and wind, but the effect of only one single substance can be tested on a particular shoot. Following the results obtained with the interveinal method mentioned on p. 177 the leaf-stalk method was used to test representative trees throughout the Research Station plantations. The injections proved that the majority of the trees were suffering from an incipient chlorosis related to iron metabolism (Roach and Levy, 1937). Accumulating evidence suggests that this condition is widespread throughout Kent and elsewhere and is likely to be of considerable economic importance.

Statistical significance of leaf-stalk method. During the past two seasons a close watch has been kept for colour or other variation in leaves which might be mistaken for the result of leaf-stalk injection. With the possible exception of an ash shoot to be mentioned later, no contrast distributed at all like the areas of leaf surface permeated as a result of a leaf-stalk injection was observed. Positive results with this method of injection may therefore be interpreted with even greater certainty than those with the interveinal method.

Although the leaf-stalk method has hitherto been applied only to the apple it is obviously applicable to other plants. A few examples will therefore be given of the types of distribution to be expected from leaf-stalk injections



TEXT-FIGS. 10 and 11. Injection through leaf-stalks which are grooved.

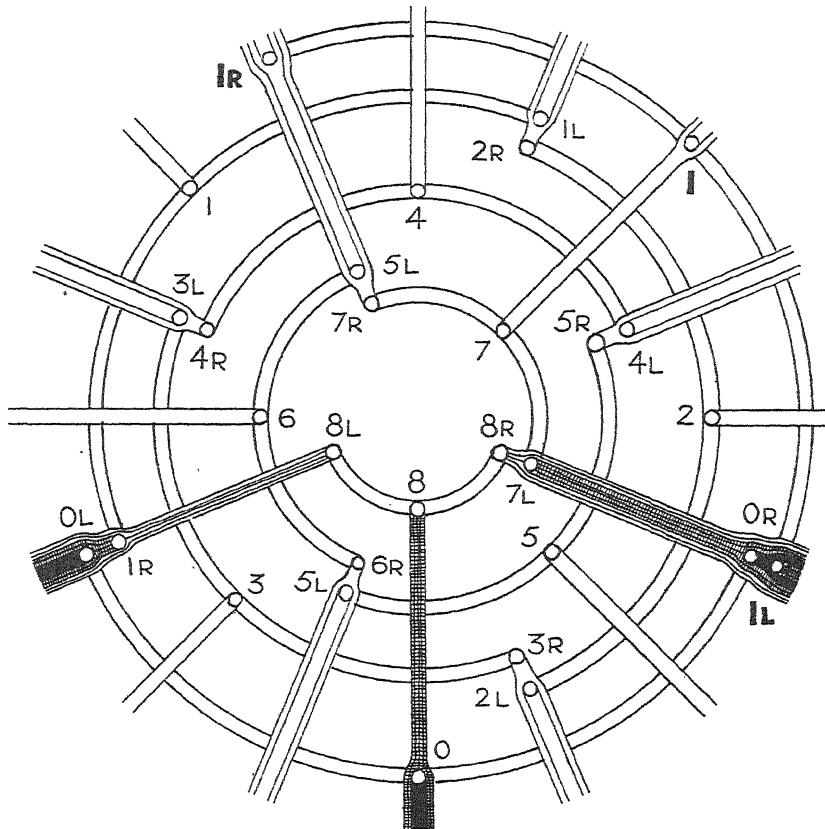


TEXT-FIG. 12. Phyllotaxis and leaf-stalk injection pattern of mature shoot. 0 is the injected leaf-stalk. Permeated areas are black.

carried out on other plants, and the manner in which the type of distribution of injected liquid is influenced by the vascular anatomy of the shoot will be touched on. The leaves of some plants have a groove running down their leaf-stalk, and the method described for the apple is inapplicable. These may be treated conveniently by one of the two methods illustrated in Text-figs. 10 and 11, which scarcely require elucidation.

(b) *Raspberry.*

The phyllotaxis of the raspberry is the same as that of the apple, namely $3/8$, but the type of distribution resulting from a leaf-stalk injection is not the same, as may be seen by comparing Text-figs. 9 and 12. The more important



TEXT-FIG. 13. Arrangement of vascular strands in mature raspberry shoot. For description see text.

differences seem to be due to the fact that the three main conducting strands which supply each raspberry leaf are so wide apart when they enter the base of the leaf-stalk that they subtend $3/8$ of the circumference of the stem, whereas the apple leaf-stalk has a single main conducting strand.

The leaf just below and the leaf just above the injected leaf-stalk have their two lateral leaflets on the side nearer to the injected leaf-stalk permeated by dye. The reason for this can be seen in Text-fig. 13, in which is indicated the distribution of the conducting strands as ascertained by stripping off the bark. The central strand of the injected leaf-stalk is labelled 0, the left-hand one OL, and the right-hand one OR; and the left-hand, central, and

right-hand strands of the leaf-stalk above and below the one injected are labelled $1L$, 1 , $1R$; $1L$, 1 , $1R$, respectively, and so on. The left-hand conducting strand of the leaf below $1L$ is immediately below the right-hand bundle of the injected leaf-stalk $0R$. The strand in continuation of $0R$, after travelling down the stem on meeting $1L$, divides into two and these pass on each side of $1L$ and of its continuation down the stem. Reference to Text-fig. 13 makes it clear that dye must have moved from the strands in direct connexion with the injected leaf-stalk (shown dead black) into those in continuation of $1L$ down the stem (shaded). The left-hand strand of the injected leaf-stalk $0L$ is immediately below the right-hand strand $1R$ of the leaf just above the injected leaf-stalk. Strand $1R$ in descending the stem meets $0L$, divides into two and these pass on each side of it. Injected liquid, therefore, is drawn, by strands $1R$, which are in direct connexion with a transpiring leaf, from strand $0L$ which is in direct connexion with the injected liquid. The permeation of these leaves resembles that of the corresponding ones on a similarly treated apple shoot in that, in both, the area at the base of each leaf on the side of the midrib nearer to the injection point becomes permeated, whereas the corresponding area on the opposite side of the midrib remains unaffected; but the actual paths are different in the two. The permeation of the next leaf up the stem differs markedly from that in the apple. In the apple, as has been shown already, rather more than half this leaf becomes permeated, as shown in Text-fig. 9, leaf 2, but the only part permeated in leaf 2, in the raspberry (Text-fig. 12) is the distal half of the second leaflet on its side nearer to the injection point. It will be seen that the right hand strand $2R$ of this leaf comes nowhere near any permeated strand; the central strand 2 is separated from the injected strand $0R$ both by the one from $7L$ and by part of the one from $8R$; and strand $2L$ is separated from the injected strand 0 by half of strand $3R$ and half of strand 8 . These facts are in harmony with the result that leaf 2 has only a small area permeated about half way along the midrib on the side nearer to the injection point. The actual type of permeation of the rest of the leaves may also be seen in Text-fig. 12 and the reason for them traced in Text-fig. 13.

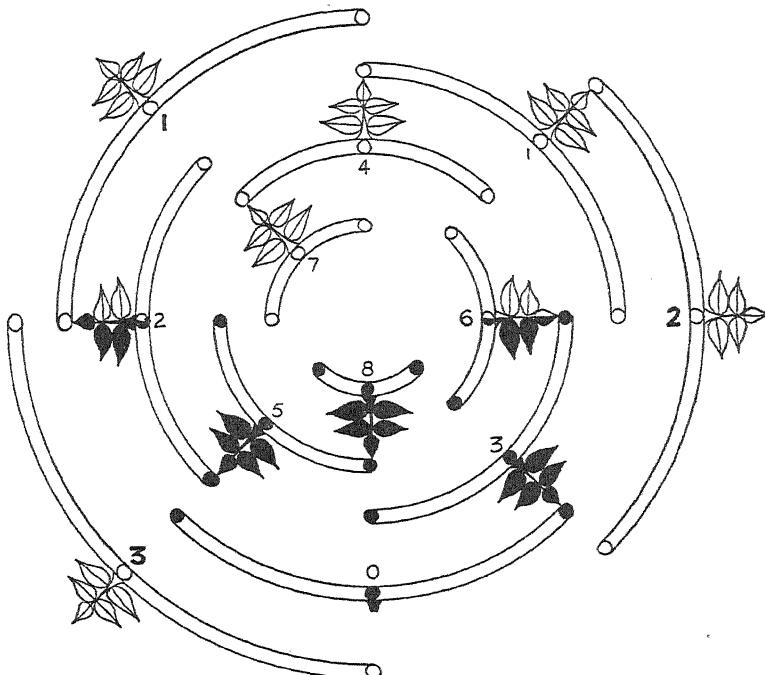
There is yet another difference between the types of permeation in the raspberry and the apple. In the latter it is the same whether an old leaf some way down the stem or a young one near the top is injected, but in the raspberry the results are quite different in the two cases. Owing to differences in the arrangement of the stems injected liquid is able to move much more freely into leaves 2 and 3 when a young leaf-stalk is injected than when an old one is so treated (for details see Roach, 1938).

An injection of the type shown in Text-figs. 12 and 13 may be useful in physiological experiments, but for the diagnosis of mineral deficiency the stalk of a just-expanded leaf must be injected, so that young leaves able to respond to the injected substance become permeated. When this is done the first and second leaves above the leaf-stalk become permeated on the side of

the main vein nearer to the injection point, and unaffected on the other. Though this type of partial permeation is not quite as satisfactory in the raspberry as in the apple, it is a useful one for diagnostic purposes.

(c) *Potato*.

The phyllotaxis of the potato, like that of the apple and the raspberry, appears to be $3/8$.¹ The potato leaf, like that of the raspberry, is supplied by



TEXT-FIG. 14. Phyllotaxis and leaf-stalk injection pattern of young potato shoot.
0 is the injected leaf-stalk. Permeated areas are black.

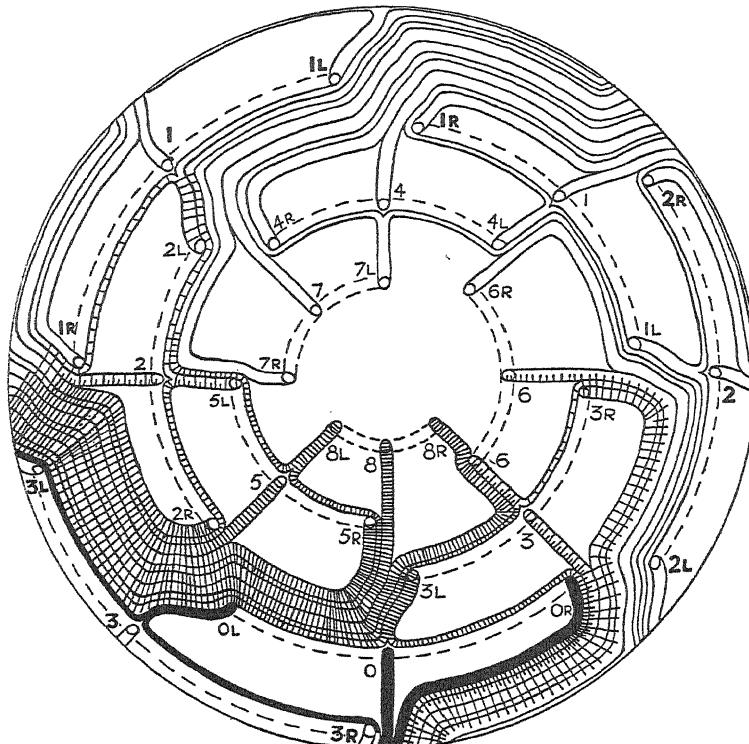
three separate conducting strands, which are so spaced as to subtend about $\frac{1}{4}$ of the circumference of the stem. Although the three conducting strands are widely separated from one another, strands from leaves higher up the stem never pass between them. A typical result of a number of leaf-stalk injections made by Hill (1938) on half-grown potato plants raised in a greenhouse are shown in Text-fig. 14, and the arrangement of the conducting strands in the stem is shown in Text-fig. 15. The injected strands are shown deep black and those supplying permeated leaves or parts of leaves are shaded, two depths of shading being distinguished. The results will be seen to differ markedly from those of the apple and the raspberry. These plants were too

¹ Artschwager (1918) states that it is $5/13$, which differs from $3/8$ by only $1/104$.

young to show the complications of structure seen in older plants (see, for example, Hegi).

(d) *Pear.*

The phyllotaxis of the pear is $2/5$, and the leaf is supplied by a single conducting strand. The types of permeation resulting from injecting an old and



TEXT-FIG. 15. Arrangement of vascular strands in young potato shoot. Lettering as in Fig. 13.

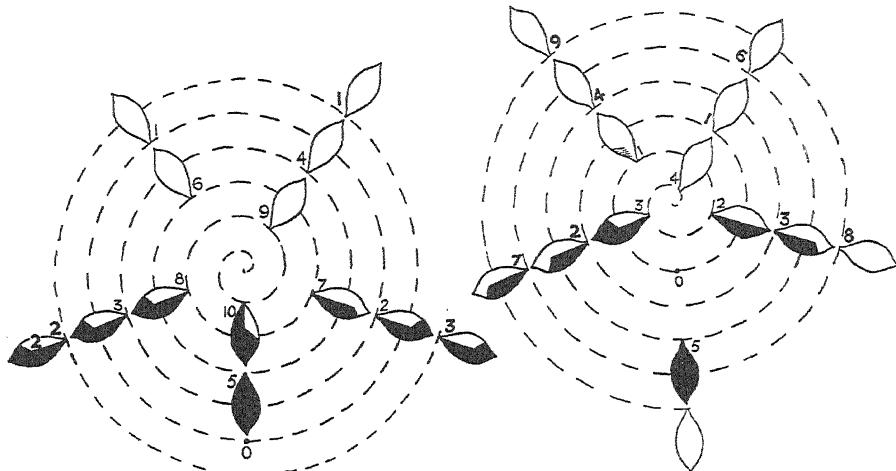
a young leaf-stalk of a pear are shown in Text-figs. 16 and 17 respectively. The latter suggests that for diagnostic purposes the stalk of the highest fully expanded leaf should be injected. The lower halves of the second and third leaves above it then become permeated on the side of the midrib nearer to the injection point and not on the other.

(e) *Red currant.*

The phyllotaxis of the red currant is $1/3$. Growing and non-growing shoots gave the same type of distribution. The third leaf above the injected leaf-stalk was the only one permeated, but in it only one sector was permeated, as shown in Text-fig. 18. The figure shows that the permeated sector is on the left of

the leaf in a shoot with a left-handed (at A), and on the right in a right-handed, spiral (at B).

All the leaves so far considered are arranged spirally on the shoot, and in botanical terminology are said to be 'alternate'. Hydrangea, coffee, and hop will serve as examples of plants with so-called 'opposite' leaves.



TEXT-FIGS. 16 and 17. Phyllotaxis and leaf-stalk injection patterns of mature (Fig. 16) and young (Fig. 17) pear shoots. ◊ is the injected leaf-stalk. Permeated areas are black.

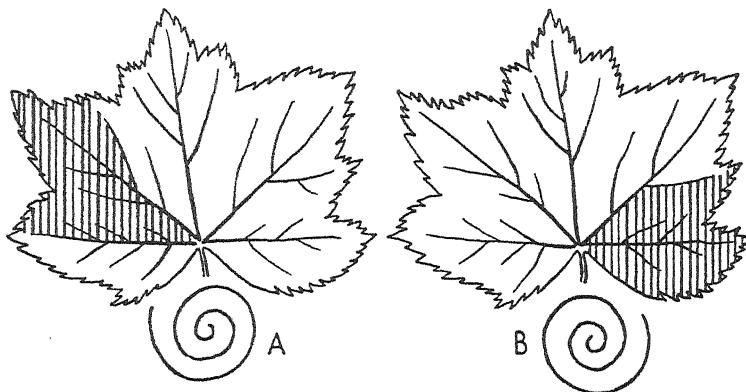
(f) *Hydrangea*.

Caldwell (1930b) has determined the type of distribution resulting from leaf-stalk injection of hydrangea the leaves of which are opposite and decussate. His results are shown in the same manner as the rest of the figures in this paper, in Text-fig. 19, from which it can be seen that in both leaves of the pair next above and below the injection point the half nearer that point becomes permeated and the further half is unaffected. Of the next pair, both above and below, the leaf immediately above the injection point is completely permeated and the opposite one is unaffected. The effect of treatment may therefore be compared in these leaves in a manner reminiscent of that obtained by Eyre and Salmon (1916) who treated one hop leaf with a fungicide and left the opposite one as a control.

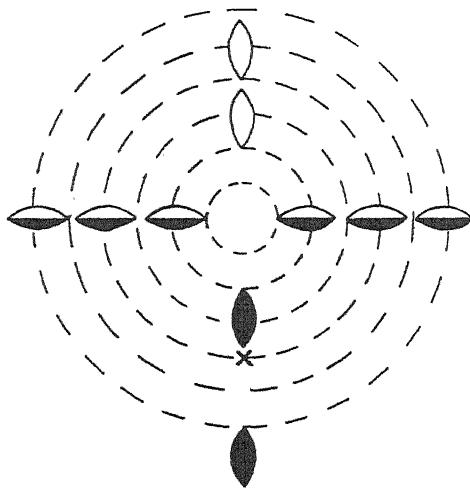
(g) *Coffee*.

Case, working at East Malling on two small coffee bushes, kindly supplied by the Royal Botanic Gardens, Kew, has recently shown that the leaves of this plant are sufficiently nearly opposite and decussate for the present purpose (see also Arndt, 1929); consequently in the pair of leaves immediately above

and below the injection point the half of each leaf nearer the injection point is permeated and the further one is unaffected, the midrib forming a sharp dividing line except sometimes near the tip. In the pairs of leaves next to



TEXT-FIG. 18. Leaf-stalk injection of red currant. Third leaf above injection point. A, a leaf from a shoot with a right-handed spiral and B, a leaf from a shoot with a left-handed spiral. Permeated areas cross-hatched.



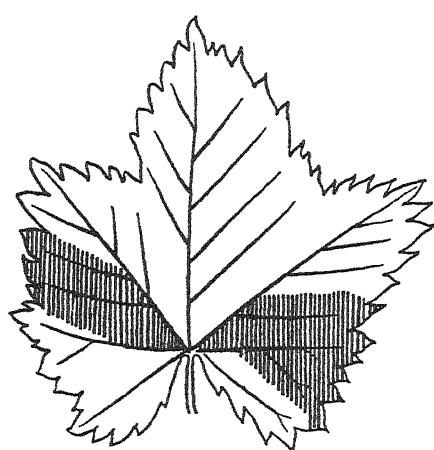
TEXT-FIG. 19. Phyllotaxis and leaf-stalk injection pattern of hydrangea. Injected leaf-stalk x. Permeated areas are black.

these, above and below them, the leaves vertically above and below the injection point are each completely permeated and the opposite leaves are unaffected. Since his return to Kenya, Case (1938) has confirmed these conclusions on the plentiful material there available. The coffee shoot, therefore, appears to be ideal for leaf-stalk injection. The leaves of the two coffee

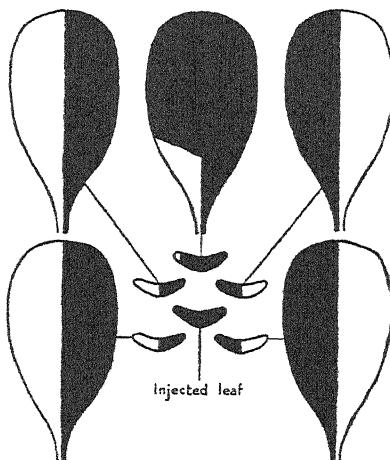
bushes at East Malling exhibited a phenomenon first described by Goppelsroeder (1889, 1901). An hour or so after injection the dye began to fade, doubtless through the formation of a leuco-compound. This, combined with the fact that the leaves were already of a healthy dark green colour, made difficult the decision as to the tissues that became permeated.

(h) Hop.

The hop, the leaves of which, as in Hydrangea and coffee, are opposite and decussate, gave an entirely different result. This is shown in Text-fig. 20.



TEXT-FIG. 20. Leaf-stalk injection of hop. Leaf opposite injection point. Permeated areas cross-hatched.



TEXT-FIG. 21. Leaf-stalk injection of mangold. Arrangement of leaf bases shown in the middle. Permeated regions are black.

Whereas in Hydrangea the leaf opposite to the injected leaf-stalk did not become permeated while those immediately above and below did; in the hop, the leaves immediately above and below did not become permeated and only the leaf opposite the injected leaf-stalk, and indeed only two small sectors of it, became permeated. It is hoped to carry out more detailed work on the hop shortly, one aim of which will be to see how the variation of leaf structure from the base of the plant to the top affects the type of injection pattern.

(i) Mangold.

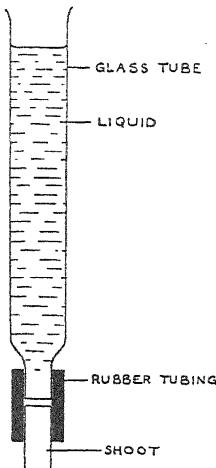
Finally the mangold will serve as an example of a plant in which the leaves arise from a crown. The result of an experiment on this plant is shown in Text-fig. 21. The two leaves above and below the injected leaf-stalk became permeated, but each only on the side nearer to the injection point.

Further work will be necessary on all the above plants, except the apple, before leaf-stalk injection work can be used in them for diagnostic purposes. In the meantime the preliminary experiments here described and discussed

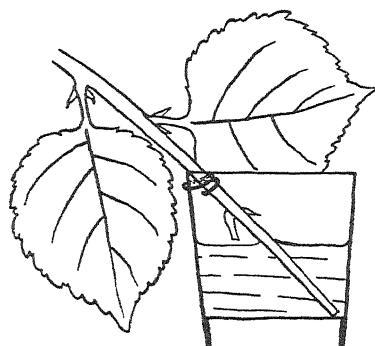
outline the field of work necessary and give some idea of the results likely to be obtained. The results so far obtained suggest that the 'injection patterns' of plants are so definite and characteristic that little difficulty should be experienced in deciding whether any particular area of intensified colour or increased growth is the result of injection or is due to chance.

V. Shoot-tip Injection

In all the methods so far described injection took place through cuts in various parts of the leaf. In the method next to be described the cut is made



TEXT-FIG. 22. Shoot-tip injection of stiff shoots.

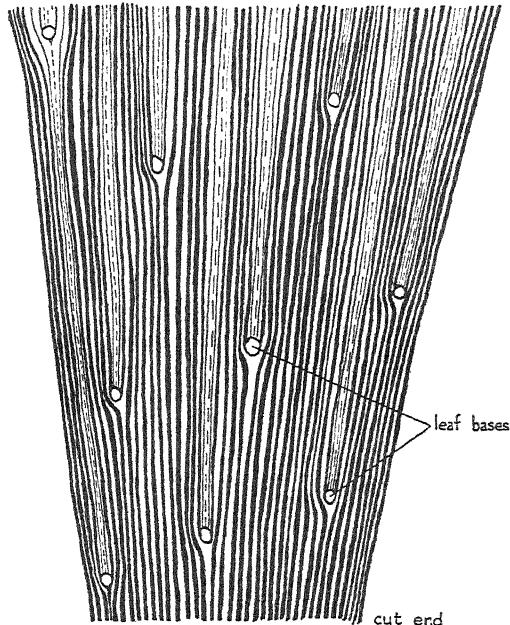


TEXT-FIG. 23. Shoot-tip injection of shoots which may be bent.

in the shoot. If the tip of a shoot be cut off and the cut end by some convenient means be kept wet with liquid, the shoot becomes injected through the cut and the material travels along the shoot and permeates one or more of its leaves. Two convenient methods of carrying out the operation are illustrated in Text-figs. 22 and 23, one for those sufficiently stiff to support a glass tube and its contents and one for shoots the ends of which can be bent. The cut must be made far enough from the growing point to open fully differentiated conducting tissues. How much to remove to permeate any desired number of leaves is best decided on the basis of preliminary experiments with dyes (see pp. 168 and 180). The removal of half an inch from the tip of a shoot which has stopped growing and is fully hardened often results in three or more leaves becoming permeated; whereas to achieve the same result in a rapidly growing shoot 3 in. or more must sometimes be removed. The removal of less than one-quarter of a current year's shoot which has stopped growing usually results in all its remaining leaves becoming permeated, and the removal of one-half results in the permeation of some of the leaves on neighbouring current year's shoots.

Localization of effects of shoot-tip injection.

The sap in all the conducting strands before they were cut was in a state of tension as a result of the transpiration pull of the leaves. In an apple tree this state of tension exists in the strands at least down to ground level (see also pp. 203 and 212). On cutting, this tension does not disappear immediately, and when the cut surface is bathed in the liquid it is drawn into the conducting



TEXT-FIG. 24. Shoot-tip injection. The lines represent the direction of the conducting strands. The heavy black lines represent strands in direct connexion with those actually cut. Those most remote laterally from the cut strands are represented by dotted lines. (Drawn from a negative kindly supplied by Dr. Storey.)

strands. This occurs whether the cuts are made under water, a method used by others whose work is considered later, or in the open air in the manner adopted by the writer. The tension is reduced to zero at the open ends of the cut strands, but as the liquid flows along the very narrow spaces it is impeded by the great friction developed, and consequently the normal tension is reduced less and less as the length of conducting tissue traversed increases. When the first leaf is reached, these conducting strands divide and pass on each side of the strands in direct connexion with the leaf (Text-fig. 24). The transpiration pull of the leaf tends to keep the contents of these strands in a state of full tension, and lying side by side with them are the permeated strands which are under only comparatively slight tension. There is, therefore, a

flow of liquid from the permeated strands into those in direct connexion with the leaf, which in consequence also becomes permeated. As already pointed out, as the liquid invades the shoot, friction impedes its flow progressively and the tension in the liquid increases; in addition, as the shoot is descended there are intercalated strands in direct connexion with other leaves, the transpiration pull of which further increases the tension. Hence the tension increases rapidly as the distance from the cut increases, and a point is reached, sooner or later according to the number of strands cut, where equilibrium is attained. The tension conditioned by the friction impeding the downward movement of the injected liquid, added to that produced by the pull of the leaves upwards, equals that in the system as a whole at this equilibrium point. The tension decreases steadily from the leaves to the roots, in which there is sometimes a positive upward pressure. While the injection is in progress liquid flows in opposite directions towards this equilibrium point wherever it may be, the injected liquid downwards and the sap upwards; but none of the injected liquid can travel beyond that point as long as the permeated leaves exert their normal pull. If these leaves are damaged, however, they do not exert their full pull and the injected liquid may then be able to travel farther into the plant, the equilibrium point having shifted.

Application of the shoot-tip method.

Three applications of this method have been described elsewhere. The first of these was in June 1932 on a single apple tree, to take advantage of an unusually heavy infection of Powdery Mildew (Roach, 1934). Ten pairs of shoots were selected so that each member of a pair was comparable with its fellow in regard to its position on the tree, vigour, severity of mildew infection, &c. One shoot of each pair was injected with water and the other with M/100 sodium thiosulphate. Six weeks later the leaves on seven of the thiosulphate-injected shoots were both more vigorous and decidedly less heavily infected than the corresponding water-injected shoots, although the condition of the members of the remaining three pairs had not altered. The chances of this result having been obtained by accident are less than one in a hundred (see below). A valuable fact, which may eventually be turned to practical advantage, was thus obtained with little expenditure of labour and without damage to the tree.

The same method was used to diagnose the cause of a severe chlorosis in a peach tree growing in a greenhouse (Roach, 1935), and more recently it has been used to prove that an, economically serious, unhealthy condition of cherry trees in the Sittingbourne district is due to faulty iron nutrition (Roach and Levy, 1937).

Statistical significance of results of shoot-tip method.

The example, given above, of the use of this method will illustrate how its statistical significance may be gauged. Three pairs of shoots were unaffected

by the disease and consequently can be ignored. In all the remaining seven pairs of shoots the disease on the thiosulphate-injected (treated) shoot became reduced whilst that on the water-injected (untreated) one remained severe. In the first pair the one disease infection has two possible hosts and the chances of its affecting the untreated shoot are therefore 1 in 2, and the same is true of the second pair; the chances of the untreated shoot being diseased in both the first two pairs is $\frac{1}{2} \times \frac{1}{2}$ and the chance of all seven untreated shoots being diseased are $(\frac{1}{2})^7$ or 1 in 128.

Had it been thought justifiable to consider all the shoots comparable with each other, the following argument would have been applicable: Of 10 untreated shoots 7 were diseased; the remaining 3 untreated shoots, and all 10 treated shoots were free from disease. If the treatment had had no effect the first disease infection could have fallen by chance on any one of 20 shoots, of which 10 were untreated, the chances, therefore, of it falling in the untreated lot being $10/20$. The second disease infection could have fallen by chance on any one of the remaining 19 shoots, of which 9 were untreated; the chances of its falling in the untreated lot being therefore $9/19$; and the chances of the first two disease infections falling by accident in an untreated lot of shoots are $10/20 \times 9/19$; and of all 7 infections falling on untreated shoots = $10/20 \times 9/19 \times 8/18 \times 7/17 \times 6/16 \times 5/15 \times 4/14 = 1$ in 646.

The same arguments would have applied had the figures referred to a colour change not outside the normal range of variation of the foliage on the branches of the tree. It is low in comparison with the results obtained by interveinal and leaf-stalk injections. The reason for this difference is that the various leafy shoots on a tree commonly vary considerably in colour and in respect to freedom from, or infection by, disease, whereas the kind of variation produced by interveinal and leaf-stalk injections is most unlikely to arise naturally.

In the other two examples the leaves on single injected shoots changed from a yellow-green to a healthy dark green which was definitely more intense than that of the foliage of any other shoot on the tree. Both the change in colour in a predictable period of time, and the mere occurrence of single leafy shoots of a healthy green colour on an otherwise yellow-leaved tree are so uncommon that a diagnosis based on the results of an injection is of high significance.

VI. *Experiments with Larger Shoots and Branches* *Branch-tip Injection*

When only about one-quarter of its length was cut from the end of a current year's shoot and it was then injected the dye completely permeated all the remaining leaves and wood of that year's growth. On tracing it backwards, its intensity decreased continuously, until at about halfway along the previous year's wood it disappeared. On entering the 2-year-old wood the dye coloured the inner and the outer of the two rings about equally, but it faded

out more rapidly in the inner than in the outer ring as it proceeded. When the injection was made halfway along the 2-year-old wood the dye disappeared in that of the previous year. When made in the middle of the 3-year-old wood the dye traversed the 4-year-old and disappeared in the 5- or the 6-year-old wood. In one branch, injected in the middle of the 3-year-old wood, the dye persisted till the 9-year-old wood was reached. The dye always lasted longest in the outermost ring of wood, next longest in the adjoining inner ring, and so on. These facts are in harmony with what is known of the structure of woody stems.

When in any of these experiments the dye encountered a lateral branch of more than about half the diameter of the one into which it had been injected, it was sometimes found to ascend the wood of the branch but along its inner side only, leaving the outer side free. Thus, it sometimes happened that about one-third of the leaves on such a branch became deeply stained while the rest remained normal. This failure of the dye to reach many of the leaves increased with the distance of the leaves from the injection point.

Injection into wood more than 1 year old is usually best avoided, because of the 'thicket' of young shoots which in response to the wounding are often caused to grow out near the cut. In practice large branches of fruit trees are occasionally shortened, as when trees are 'dehorned', and if the remaining stumps of these happen to be furnished with large numbers of small shoots (fruiting spurs), these would form suitable material for branch-tip injections. As will be seen later, should more than about half the length of such large branches be removed, the injected liquid will reach the main crutch. All the branches may be injected, if desired. Thus, a control one may be injected with water only, and each of the others with a different liquid without any risk of mixture. Any branches left uninjected however, will absorb liquid from any of the others with the conducting strands of which their own are united. Further, if the soil is dry, those portions of the root system in direct connexion with individual branches will also become permeated, each with its own liquid.

If one main branch be cut off, leaving a stump which is then injected by a suitable method (to be indicated later), the liquid will enter those branches the strands of which come into contact with those of the injected branch in the crutch and trunk. This matter will be discussed more fully later (see p. 202). When the injection strands come into contact with those of all the other main branches, such an injection may result in a practically uniform permeation of the whole tree.

Application of branch-tip method.

An actual application of this type of injection, carried out by the writer in co-operation with H. Wormald, will serve as an example of how it may be applied usefully to more practical problems. It is customary to remove branches of plum trees suffering from Silver Leaf disease as soon as many

of the laterals begin to die. The causative fungus (*Stereum purpureum*) is sometimes localized in the branch and is entirely removed with the branch, but sometimes it extends into other branches. Under the latter conditions the removal of such a branch presents an opportunity for injecting a fungicide into the wood so far as to surround completely any still localized fungus growth which may remain in the wood. Moreover, if the branch is large enough, and if its actively conducting strands unite with those of all the other branches in the main stem, it may be used for injecting the whole tree with a nutrient solution in order to stimulate it to 'grow away from' the fungus. Indeed, the fungicide and the nutrient may be combined in a single solution. The work in progress on these lines is an extension of that of Brooks and Bailey (1919), who, as already stated (see p. 165), injected fungicides including 8-hydroxyquinoline potassium sulphate through root stumps cut for the purpose.

VII. Leach's Shoot Injection Method, and the Branch Injection Method of Collison, Harlan, and Sweeney

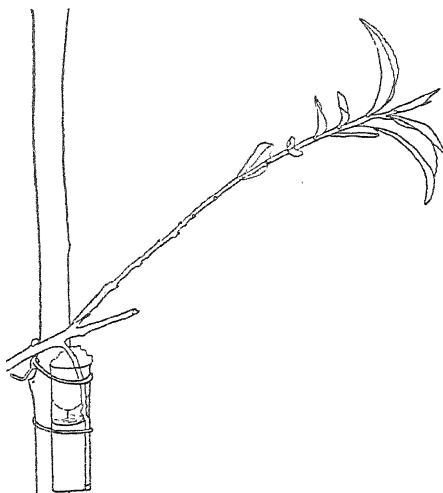
In joint work with Storey (Storey and Leach, 1933, Storey, 1938) Leach evolved a simple and effective method for shoot injection which has already been mentioned on p. 166, and is illustrated in Text-fig. 25 which has been drawn from a negative kindly supplied by Storey. This suggests that these workers used shoots of approximately equal sizes for injection by immersion in a liquid and for permeation. Experience with apple and other trees has shown that the cut shoot immersed in the liquid must be at least as large as the one to be permeated, otherwise permeation will not be complete. Further, if this condition is satisfied, the method should be applicable to shoots and branches of any size. As applied to larger branches, this method with slight modifications becomes that of Collison, Harlan, and Sweeny (1932) who, as already stated, observed that the injected and the permeated branches needed to be about equal in size. The general paths of distribution of the introduced liquid along the shoot below that used for the injection are similar to those in leaf-stalk and shoot-tip injections of small branches. For larger branches distribution follows the paths described in a subsequent section, which deals with the injection of branches by a method applicable to almost any tree.

Application of Leach's method.

This method was used by Storey and Leach on small shoots of the tea bush, and enabled them to show that a chlorosis of great economic importance was due to sulphur deficiency. It has a range of applicability similar to that of the shoot-tip method and has the advantage of retaining the sensitive expanding leaves at the growing tip. Its field of use, however, is somewhat limited by the necessity of using two shoots about equal in size arising near the branch tip. There is no difficulty in practice in deciding which is the better method for a particular problem. In both methods account has to be taken of the

stimulus given to the growth of other parts of the tree by the removal of a piece of a shoot or a branch. This removal of part or practically the whole of a shoot or branch, necessary in both methods, limits their use to small

scale diagnostic injections, except occasionally when for special reasons larger branches may be removed.



TEXT-FIG. 25. Leach's method for shoot injection.

pruned. As a result of this their tops consist of a varying number of main branches, usually ten or more, often very similar to one another. These lend themselves readily to injection experiments in which several branches can be treated, each in a different way, so that a comprehensive experiment can be carried out on a single tree. Each main branch on such a tree has a considerable number of small branches arising from it at more or less uniform distances along its whole length. With such material the range of penetration following injection may be increased beyond that usually possible with the branch-tip or the Leach methods. For this purpose a solution of the substance to be tested is held in a reservoir A (Text-fig. 26) attached to the branch B, and the solution flows thence through rubber tubing C under a few inches head of pressure into an injection hole D, drilled diametrically through the branch, and of a bore equal to about one-eighth that of the branch or one-twenty-fourth of its girth.

Distribution and localization of injected liquid.

As is well-known, the pull of the leaves on the fluid in the woody tissue of the tree keeps it in a state of tension; consequently, the injecting liquid rushes up the opened conducting channels and so passes eventually into the leaves, provided, of course, that the experiment is done when the tree is in leaf and transpiration is active. These results and those which follow are readily

Application of the method of Collison, Harlan, and Sweeney.

When large branches are to be injected use is generally made of a method, to be described in the next section, in which no appreciable mechanical damage is done to the tree; but where there is no objection to the removal of a considerable portion of a tree the branch method of Collison, Harlan, and Sweeney may be used.

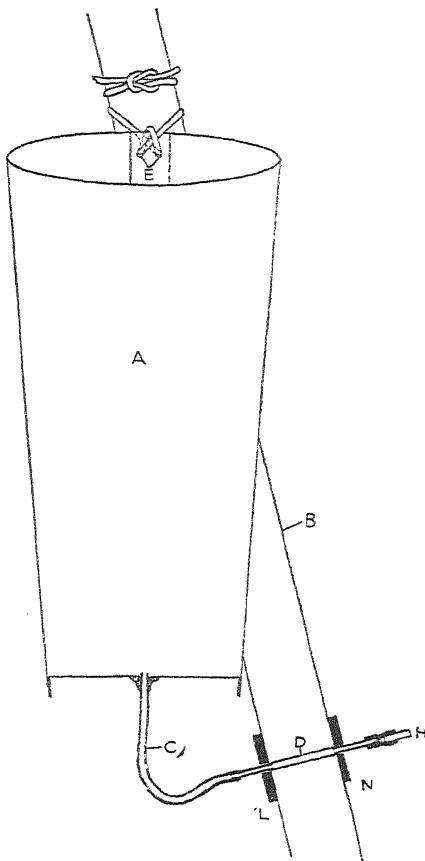
VIII. Injection of Individual Branches

A considerable proportion of fruit trees in certain countries are spur-

demonstrated by injecting a suitable dye solution (see p. 214) and later on removing the bark from the stem and so laying bare the surface of the wood. The rate of travel upwards varies but is of the order of $\frac{1}{2}$ in. per minute. The dye also travels circumferentially round each annual ring of the wood at the rate of about $\frac{1}{20}$ in. per minute, until the whole circumference of the branch at the level of the hole becomes permeated. Accordingly, soon after the injection has been started every leaf above the hole draws on the coloured liquid; and, during the progress of the injection the flow of sap is replaced by that of injected liquid.

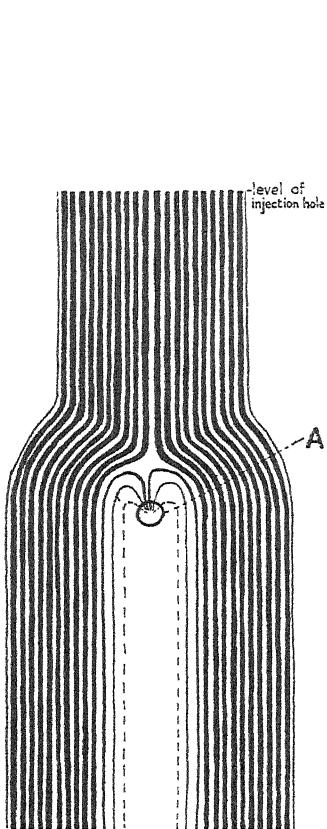
The injected liquid also moves down the branch at a rate which at first is about equal to that of the upward movement. When dye is injected so that the progress of the injection may be watched and the wood laid bare by carefully removing the bark, two bands of colour, each of a width equal to the diameter of the hole, are seen to start one from each end of it and travel down the strands of which the wood consists. As the dye travels around the circumference of the branch these bands broaden until they meet and then the whole circumference has become permeated. When the first side-branch (A in Text-fig. 27) below the hole is reached, the colour is seen to branch, follow the strands, and pass down on each side of it, leaving temporarily uncoloured

those (left white or drawn lightly in the figure) immediately below the side-branch and in direct connexion with it. In Text-fig. 27 one is supposed to be looking straight at the stump A of the side-branch, which has been cut off just above the swelling where it joins with the main stem. While injection is going on, transpiration by the leaves borne by this side-branch tended to keep the strands in direct connexion with it in a state of full tension, whereas the tension in the permeated strands is reduced by the inflow

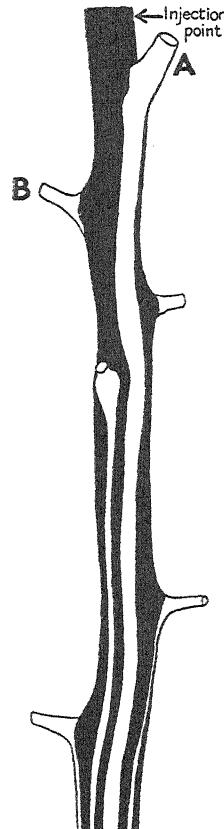


TEXT-FIG. 26. Method for injecting small branches and trees. The liquid is held in a milk carton A, attached to the branch B, and led through rubber tubing C, to the hole D, which passes through a diameter of the branch. For further explanation see text.

of injected liquid; coloured liquid, therefore, flows from the first strands into the second and a band of colour is seen to ascend the side-branch on its side nearest to the main branch. This band broadens and, if the diameter of the side-branch is not more than one-quarter that of the main branch, will



TEXT-FIG. 27. Arrangement of strands. For description see text, p. 199.



TEXT-FIG. 28. Permeation of stem. For description see text, p. 200.

become completely permeated. The same fact is illustrated in Text-fig. 28 which represents a short length of spur-pruned branch in which the strands in direct connexion with side-branches are left white. The whole stem at the level of the injection point is permeated and is drawn black. As in Text-fig. 28, when the first branch A is reached the injected strands divide and flow on either side of the side-branch. The same thing happens as each succeeding side-branch is reached so that when the second side-branch B is passed, the originally undivided cylinder of injected strands becomes separated into two, when the third is reached into three, when the tenth is reached into ten parts,

and so on. The flow of the injected liquid is impeded by the high resistance offered by the walls of the fine tubes along which it travels and it is being drawn on by more and more absorbing side-branches as the main branch is descended. Further, the normal sap will flow upwards as soon as a certain suction is established. It is obvious, therefore, that a point must soon be reached beyond which the injected liquid is unable to travel. As might be expected this point is reached more quickly in a spur-pruned main branch which has on it many side-branches than in a main branch which has few side-branches or none at all.

Position of hole.

When the branch is well furnished with small side-branches, such as fruiting spurs on a spur-pruned tree (Text-fig. 29), the injected liquid does not travel more than one-third as far downwards as upwards, in which direction, as already stated, it reaches the tip. In such material, therefore, the hole is bored at a point, such as one-quarter the distance from the desired limit of penetration, viz. from the crutch to the branch-tip (see x of Text-fig. 29). When the base of a branch is not well furnished with side-branches the hole must be made higher up; and when a branch has no side-branches on the lower half of its stem it may safely be injected half-way between the desired lower limit of penetration and the branch-tip. Text-fig. 30 illustrates such a branch. The injection point is at x; heavy permeation is indicated by deep black, and light permeation by shading.

Application of branch injections.

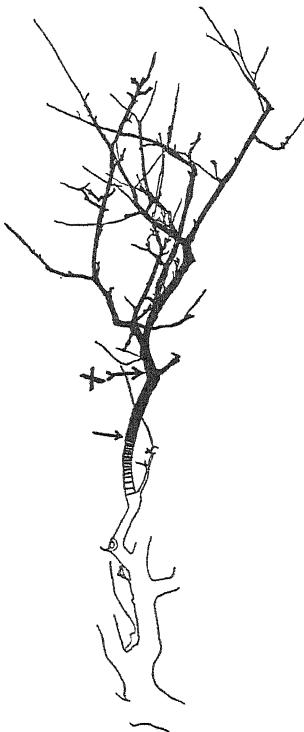
Many trees in commercial plantations have ten or even more comparable branches which may be injected each as a separate unit. Each such unit may bear growths of various kinds, for example leader shoots, dards, fruiting spurs, and fruit. The method, therefore, is convenient for testing the general effects of a substance, such as an artificial fertilizer, not only on the total amount of growth but also on how this growth is distributed. For example, one substance or mixture may tend to encourage 'woody' leader growth, which rarely bears fruit, whilst another may encourage the types of growths which normally bear fruit. Further, the effects on the amount and quality of fruit produced may also be studied, including its storage quality and its resistance or susceptibility to disease attacks.

In two preliminary notes, already published, the effects of injecting certain artificial fertilizers into the branches of spur-pruned pear-trees (Srivastava and Roach, 1937), and into those of apple-trees which had not been spur-pruned (Sen, 1937) were described. Sen also injected sugars in his work, which formed part of an investigation concerning biennial bearing. A. S. Horne of the Imperial College of Science and Technology, London, is at present collaborating with B. F. G. Levy and the writer in an investigation of the effect of the injection of mineral fertilizers and other substances on the

susceptibility of the fruit of the fruit to rotting by a fungus of economic importance. H. Hill has used the method for testing in a preliminary way the effects of certain of the chemical elements that normally occur in fruit trees in minute



TEXT-FIG. 29. Injection of branches with many side-branches. X marks suitable injection points. Right-hand branch untreated, left-hand branch injected with a complete nutrient (0.5% urea + 0.5% dipotassium hydrogen phosphate).



TEXT-FIG. 30. Injection of branches with few or no side-branches near their base. X is suitable injection point.

amounts only. The results he has obtained with a nickel salt suggest that further trials with this element are desirable.

IX. Injection of Individual Branches together with their Roots

As already stated, in the injection method just described the liquid travels not only into the branch above the injection point but also to a less extent downwards; if the hole is placed low down there is thus a risk of liquid leaving the branch under examination and entering those contiguous to it. The lower the hole is placed on the branch the greater is the amount of liquid which enters other parts of the tree. Not only does it invade other branches, but if

it is close to the main crutch and the soil is dry the liquid enters the roots, sometimes penetrating even the finest rootlets at the ends of the longest roots (see also p. 212).

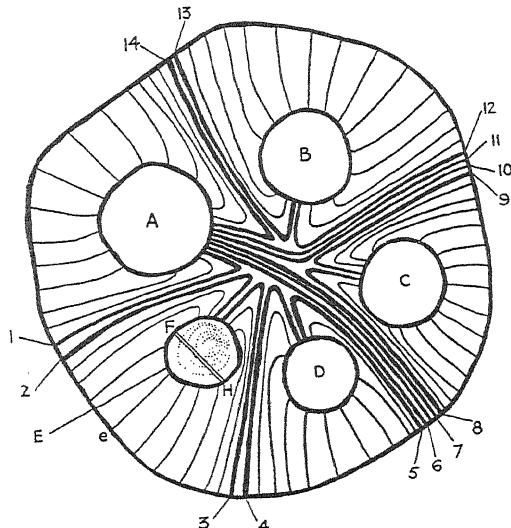
The planning of the type of injection to give the desired distribution depends, as in the types previously considered, on an accurate knowledge of the structure of the parts concerned—in this instance the main crutch. Two simple experiments will make this point clearer.

If dye solution be introduced into a shallow hole bored into the wood of an unbranched stem of a tree it travels mainly upwards and downwards along the length of the stem; but it also travels a short distance circumferentially, and a still shorter distance radially. The relative rates of travel in these three directions have been found experimentally to be of the order of 100 : 10 : 1. Further, if the injected stem be allowed to dry the resulting cracks run in the same direction as the dye; if the wood be split the splits also run in the same direction, and if thin strands are detached these too follow the same course. Since injected liquid follows the woody strands so closely, their distribution in the main crutch must largely determine the type of distribution of the material supplied by injections in its vicinity.

The arrangement of the woody strands in the main crutch may be demonstrated by an experiment which is a development of the one just described. Instead of a single shallow hole a sufficient number are made at $\frac{1}{2}$ -in. intervals to encircle the whole main stem of the tree, and into these differently coloured solutions are injected for half an hour—a length of time sufficient if the day is fine and the soil dry. The tree is then dug up and its bark removed. As a result of the closeness of the holes to each other circumferential movement of liquid is less than when a single hole is bored, but longitudinal movement is not appreciably less. The whole stem of the tree show bands of colour, each about half an inch in width, which run from the roots along the main stem into the branches.

Text-fig. 31 is a diagram of what may be seen looking vertically downwards on the main crutch of a bush-trained tree treated in this way. The five branches have been cut off just above the crutch and the cut ends of their stumps are seen at A, B, C, D, E. The main stem is drawn as if expanded at the base, where it has been cut off. The heavy lines 1, 2, &c., running from the cut ends of the branches to the base (i.e. the periphery of the figure) indicate the main directions of the woody strands. Strands 1 and 2 lie side by side in the main stem and also as they pass between branches A and E, but when they nearly reach the centre of the main crutch they bend, 1 going into A and 2 into E. The upper bent part of strand 2 lies side by side with that of strand 3, the lower end of which lies next to strand 4, which travels into branch D. As already stated, injected liquid moves most rapidly along the woody strands, but it also travels quite appreciably from strand to strand circumferentially. It is apparent, therefore, that liquid injected through a hole which cuts through strands 1 and 2 will enter not only branches A and E

but also branch D. Strands such as these must therefore be avoided when the injected liquid is to be confined to a single branch. In other words, when the hole is made in the branch it must avoid the part nearest to the centre line of the tree if the injection of other branches is to be prevented; and if it is



TEXT-FIG. 31. For description see text, p. 203.

made in the main stem it must avoid the junction between two branches or any point immediately below it, allowance being made for any twist in the stem. The risk of the injected liquid travelling into other branches than those desired may be reduced to a minimum by boring a single shallow hole as far away as possible from these strands, as at E, Text-fig. 31, on the outside of the branch. Such an injection through a single shallow hole does not, however, always result in a complete permeation of the whole branch. When this is desired the hole is made as shown at FH, for it has already been shown that injection through such a hole usually results in a complete permeation of the whole branch. There is a slight risk of liquid travelling from the hole circumferentially to strands 2 and 3 and thus out of the branch E into branches A and D. This risk is greatest in the inner annual rings since they are smaller, and so the actual linear distance to be travelled by the liquid from the hole to strands 2 and 3 is smaller. This risk is lessened by the fact that the branches grow eccentrically, the rings being thicker on the outside than towards the centre of the tree, with the result that the hole, even if drilled diametrically through the branch, does not pass through the oldest rings at all. The hole, therefore, may be made with advantage slightly to the outside of the diameter and thus miss as many as possible of the oldest rings. All rings except the oldest one or two in twenty-year-old apple-trees conduct

injected liquid, but in the plum, and other trees which have a core of dead non-conducting heart wood, this precaution is not so necessary. Risk of leakage from one experimental branch to another is further lessened by the fact that each is being injected simultaneously and thus the tension in each is being reduced by approximately equal amounts. Untreated branches should, for this reason, be injected with water. Branches B, C, D, and E are alike in that the strands in continuation of them in the main stem are undivided, and all of them can be treated as just described. The larger branch A, however, possesses more than one set of strands in the main stem; in addition to the strands bounded by 1 and 14 it has two small bands, 6, 7, passing down into the main stem between branches C and D, and 2 smaller bands still, 10, 11, between branches B and C. If branch A is left uninjected bands of strands such as 6, 7 and 10, 11 will tend to prevent liquid passing from D to C or from C to B, and vice versa. Such a branch is known as a 'leader'; it is recognized by the fact that it is usually larger than the other branches, and commonly, but not always, arises nearer the centre of the tree. Strands such as 6, 7 and 10, 11 also may usually be recognized at sight; thus branches such as A and B, D and E, E and A, which respectively have no such strands between them, meet each other at the crutch at a sharp angle; but branches B and C, C and D, either meet each other in a smooth curve or are separated by a flat area of bark obviously connected with the leader branch.

The tree just described as an example is intermediate between those resulting from two types of training. The first is the well-trained bush tree common in the Eastern fruit-growing district of this country, in which the ideal is main branches of equal sizes, that is for all leader dominance to be counteracted so that neighbouring branches actually meet each other in the crutch and are not separated by woody strands coming from a leader branch. In injection work it is particularly important in such trees that all untreated (control) branches which are to be used for comparison with those injected with nutrient or other substance, should be injected with water, to prevent them from withdrawing liquid from those specially injected. For the same reason the injection of all branches must be begun and stopped at about the same time, any of the reservoirs containing injection solutions becoming empty before the end of the experiment must be refilled immediately with water.

The second type is the well-trained modified leader tree common in Canada, in which neighbouring branches are always separated by strands from the leader branch. When these separating strands are well developed they constitute a sufficient barrier between the branches, and those 'untreated' need not be injected with water. The leader branch will tend to absorb a little of each of the injected liquids, but, apart from this, it is of so much greater vigour than the other branches that for experimental purposes it is not comparable with them. Untrained or badly trained trees have to be taken on their merits, some being suitable for experiments of this kind and others quite unsuitable.

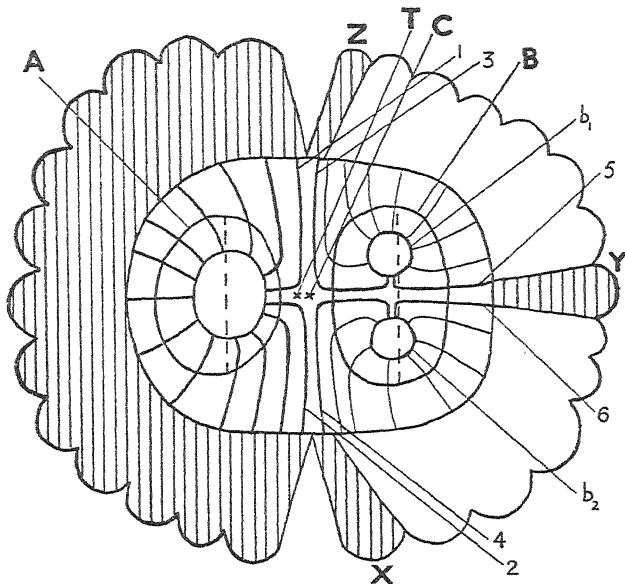
In modified leader and in untrained trees, where strands from the leader branch separate all the injected branches from each other, the way in which the strands associated with these injected branches subdivide just above the crutch is of little or no importance from the present point of view, but may be of the utmost importance when bush-trained trees are being treated.

Structures which make difficult the injection of individual branches and their roots.

Two examples will be given of branch structures the injection of which results in distribution of liquid in a way which may be most inconvenient from the experimental point of view. The first was discovered as the result of an early experiment carried out on a plum-tree. This tree had two branches A and B (Text-fig. 32) nearly equal in size. These were injected through holes, indicated by dotted lines, A with 0·1 per cent. light green and B with 0·1 per cent. ponceau red 3R. Since light green travels about $1\frac{1}{2}$ times as fast and $1\frac{1}{2}$ times as far as ponceau red, it was expected that the parts z, x of branch B nearest to A would become dyed green, but, in addition, a by no means negligible amount of foliage, y, on the side of B opposite to A also assumed the metallic green colour characteristic of the light green dye. This result was at that time quite unexpected. Removal of the bark and partial dissection of the branches made the reason clear. Branch B, just above the crutch and the injection hole, divided into two sub-branches b_1 and b_2 which were in a plane at right angles to that of AB. It will be seen from the diagram how the free-moving light green dye solution passing down strands 1 and 2 on the side of branch A next to B could move into strands 3 and 4 respectively, since these lie side by side in the main stem. Having invaded strands 3 and 4 the dye ascended in them to the crutch b_1 and b_2 and then ascended on the inner sides of both these branches. In a similar manner it crossed into strands 5 and 6 and descended them for some distance. The dyed foliage y was carried by a small branch which happened to arise on strands 5 and 6.

Should this branch-injection method be employed experimentally occasional results of this kind are to be expected and the question will arise as to whether the change occurring on a particular branch or part of a branch on a remote part of a tree can have been influenced by a liquid travelling from a distant injection hole. Fortunately such a question can be decided without seriously damaging the tree. Using the same diagram for illustration, any liquid travelling from any part of branch A into any part of branch B must pass the centre c of the main crutch. A hole $\frac{1}{32}$ in. in diameter is therefore drilled at the point T through the bark so as to penetrate say $\frac{1}{8}$ in. into the wood. Dye solution is dropped into this hole and into it is inserted a suitably tapered glass tube into which enough solution may be introduced to last for, say, one hour. This dye will mark out the region most likely to be affected by any injection carried out anywhere in branch A. The course of the dye can be followed by making as thin and neat a cut as possible in the bark of branch B,

a foot or so above the hole T along the suspected route, until the dye is recognized; the process is then repeated. An alternative method is to allow the dye injection to proceed until the colour of the dye is recognized in the



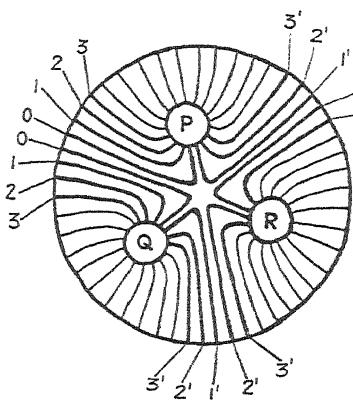
TEXT-FIG. 32. For description see text, p. 206.

leaves. If dyes such as acid fuchsin or light green or patent blue are used little damage need be done to the branch. Other and equally inconvenient types of distribution of strands have been observed in a number of trees and are described elsewhere (Roach, 1938).

Influence of structure of root crutch on the possibility of injecting individual branches and their roots.

So far, attention has been concentrated on the main crutch, that is, on the region where the trunk divides to form the chief branches; the structure of the corresponding region where the trunk divides to form the main roots is also of importance. The same general considerations hold for this region as for the stem crutch. However, there is not a root corresponding to each chief branch and the roots seem to divide quite independently of the division into branches. In consequence the end of a hole placed in a satisfactory position with regard to the visible branch crutch may be in the worst possible position in regard to the usually invisible root crutch. It may, for example, cut strands which pass down to the junction of two main roots, and if this happens there is a risk of the liquid passing into the other main branches of the root, as may be seen in Text-fig. 33, in which the two strands o are actually cut by the

hole (at a point higher up the trunk and not shown in the diagram), and 1, 2, 3 become permeated by circumferential movement of the liquid in the main stem, and 1', 2' and 2', 3' by circumferential movement in roots P and Q, respectively. The main groups of strands between roots P and R and R and Q



TEXT-FIG. 33. For description see text, p. 207.

respectively may both connect up with main branches other than the one intended, that is, the one 'above' strands o. The actual volume of liquid leaving such a branch will be slightly less than with a corresponding 'leakage' across the branch crutch because the resistance of the whole length of the trunk will tend to impede its flow. The risk of this leakage will be less in a 'standard' tree with a long trunk than in a 'bush' tree with a short trunk. Should leakage of this kind take place it will be greater the drier the soil is, because, as seen later (p. 212), the movement of injected liquid into the roots is greater the drier the soil. Further, if one root is in drier soil than the

others, more liquid will flow into it than into them. One consequence is that the amount of leakage from a given injection operation is dependent to a slight extent on the relative degree of dryness of the soil surrounding the various roots, but this is a point of little more than academic interest. A test for a suspected leakage of this kind can be made by means of a small-scale, dye injection, the hole being made in healthy wood immediately below the main injection hole—allowance, of course, being made for any twist in the stem—and as close to it as possible. Strands such as the two labelled 1' between P and R, and Q and R respectively are bound to be permeated, if any are, both by the experimental liquid and by the exploratory dye solution, but the two members of each of the two pairs of strands such as those labelled 3', on the edges of the 'leakage regions', may be respectively the one permeated by the experimental liquid and the other by the dye if the relative distribution of soil moisture was very different on the two occasions. A somewhat larger injection hole may therefore be necessary to test for leakage across a root crutch than for one across a branch crutch.

Application of the method.

As already stated, this method is particularly suited for experiments in which it is desired to inject some of the roots themselves as well as a branch. Otherwise it has no great advantage over the last one in which the branches alone are permeated and not the roots, and it has certain disadvantages. It has been described in detail not only because it has a definite although restricted

field of use, but also to make clear its disadvantages and how they may often be avoided. The methods so far considered were designed almost entirely for diagnostic and analogous experimental work. The remaining method—that for injecting whole trees or plants—may also be used for diagnostic and experimental purposes, when it is desirable to compare with each other units as large as whole plants, but in special circumstances it may also be used for treating whole plantations on a commercial scale.

X. *Injection of Whole Trees*

In the injection methods described in the earlier part of the paper the first and most essential object was to confine the introduced liquid within definite limits, and the same was true for the branch injections described later except that the size of hole had to be suited to the size of the branch in order to ensure that every leaf above the hole became permeated. When whole trees are to be injected, on the other hand, the primary consideration is to ensure that every branch shall have its full share of the liquid.

The methods vary according to the size and the shape of the tree. For trees trained as cordons the method for branch injection (pp. 198–202) is quite satisfactory except that the hole should usually be bored a little below the level of the lowest branch. The same apparatus and procedure are suitable for bush trees with trunks up to 2 or 3 in. in diameter, except that the hole must be placed in definite relation to the branches. This point will be considered in the following section. Practical details are given in another paper (Roach, 1938).

Position of hole.

The correct location of the hole can be decided by referring once more to Text-fig. 31 already used in discussing the best position of holes through which to inject individual branches and their roots. For that purpose the strands indicated as thick heavy lines with numbers against them had to be avoided; since the inner strands of all the branches either meet or come close to each other in the centre of the crutch, if one of them is injected there is a tendency for them all to become permeated. But what was undesirable for that particular purpose is highly desirable, indeed necessary, for the present one, although even these strands vary somewhat in usefulness for the object now in view. Liquid injected through a hole that cuts strands 1, 2 will pass directly into branches A and E; moreover, because the part of strand 2 in branch E lies side by side with strand 3, the lower part of which in the main stem lies side by side with strand 4 which in turn passes up into branch D, this branch also will become permeated. Liquid injected into strands 9 and 10 passes directly into branches C and A, across, into and up strand 11, across this strand into strand 12, and so into branch B. Some hundreds of experiments have proved that injections through holes placed in such a position lead to practically uniform distribution of liquid throughout the branches, more

uniform, in fact, than sometimes results from the application of manure to the soil and its natural absorption by the roots (Roach, 1935). The direction of the hole should divide the tree into as nearly equal halves as possible. From the work done the impression has been gathered that strands, such as 6 to 7, coming from a leader branch, tend to draw the liquid more strongly than do other branches. Although no definite proof of this can be given, it is safer to avoid such strands as far as possible, and when the hole has to be made under a crutch entered by them, such as the one between B and C, it is best to inject to one side of them, namely, as already suggested, across 9 and 10, rather than parallel with them.

The principles underlying the proper placing of the injection holes have already been described and the desirability of placing them as nearly as possible in the ideal positions has been emphasized. A comparison of the results of one experiment carried out before these principles were worked out with those of one carried out after they had been elucidated is instructive.

An early faulty experiment.

A 13-year-old apple-tree was bored through its main stem just below the crutch with a $\frac{1}{4}$ in. bit, and the hole was connected with rubber tubing to a reservoir containing 10 litres of 1·01 per cent. solution of potassium nitrate. The whole of this was absorbed during the hot dry afternoon of 17 June 1932. Scorching of much of the foliage was apparent later the same evening, but was much more evident on the following day when the affected areas of the leaves had changed from 'dried up' green to brown. This scorching will be dealt with in greater detail later on. It was most severe in the leaves of two branches directly above the two ends of the injection hole, and least so in two branches most remote from the hole.

Two months later the new leaves that had developed were large, thick and of a strikingly healthy, dark green colour all over the tree, in marked contrast with the small, thin, old leaves of unhealthy colour on the tree prior to the injection. The whole tree from being the most unhealthy looking in the whole plantation had been changed into the healthiest looking. In the autumn the extension growth both for 1931 and 1932 was estimated on nineteen shoots which had remained unbranched in both these years. The results were as follows:

					Ratio
Total extension growth (cm.) of 19 shoots.	1931.	1932.	1932/31.		
8 shoots on two branches over hole	161	287	1·8		
5 " " one branch nearly over hole	120	207	1·7		
7 " " two branches remote from hole	172	256	1·4		

Thus the two branches, the foliage of which was most severely scorched immediately after injection, eventually increased most in length, whereas those the foliage of which was least damaged eventually increased least in length. Hence the same lack of uniformity of distribution of the solution

along the branch was indicated both by the immediate damage produced and later by the increase in growth. This is an example of the most unsatisfactory distribution ever likely to result from an injection through a hole made in the wrong position in a tree with five main branches arising from the main stem at about the same height. The lack of uniformity in the distribution resulted almost entirely from the hole being directly beneath two branches instead of being between them and rather low down below them.

A later experiment with holes correctly placed.

As an example of the type of results obtained when the holes are properly placed one may be mentioned which has already been the subject of a Progress Report (Roach, 1935a). In this experiment eight, twenty-one-year-old bush apple-trees in a commercial plantation were injected with a solution containing equal weights of dipotassium hydrogen phosphate and urea at rates which varied between 10 and 50 lb. per acre for each substance. After the injection one branch of each of three trees showed an easily visible but not serious amount of leaf damage, and two others were slightly damaged; in the remaining five trees damage was negligible. In each of the first three trees the end of the hole was not accurately on the dividing strands between two branches but was on the damaged side of the branch. The main stem of each of these three trees, which was about 10 cm. in diameter, was bored with a single hole which passed diametrically through it, and the slightly faulty results were due to the difficulty of 'aiming' at an invisible point on the farther side of the stem. This difficulty does not arise in the newer method described on p. 209.

In the autumn differences in growth were apparent between these eight trees, eight other comparable untreated trees, and a further eight trees in the same plantation which were injected with water only. These varied from a barely detectable difference in growth in the tree receiving the smallest amount, to a fourfold increase of growth in the one that was injected at the rate of 27 lb. per acre. As far as could be judged from a careful visual examination of the injected trees all the branches on each were equally invigorated.

The imperfect distribution of the potassium nitrate in the first experiment, as judged by the damage and the subsequent growth response, is typical of the irregular distribution of dye and of various salts (as judged by the leaf damage) in many scores of the earlier experiments carried out before the importance of the position of the hole was realized. The results of the more correct experiment just described are typical of those obtained in some hundreds of the later injections. The positions of the injection holes must therefore be selected with care if the best results are to be obtained.

Exclusion of air from injection hole.

In this section will be considered a precaution which has been exercised by a number of past workers but the importance of which is still not established. In transpiration experiments, &c., it is usually recommended to cut

off the plant organ under water to prevent the entrance of air into wood vessels. Shevyrev (1894) seems to have been the first to advocate the exclusion of air from cut surfaces that are to be injected; but it has been shown (see below) that his reasons are not conclusive, and he does not appear to have submitted them to definite experimental tests. This is true also of a number of other workers, e.g. Mokrzecki (1903), Rumbold (1915, 1920), and Storey and Leach (1933), who have followed his lead in this respect. Roth, on the other hand, as already pointed out, obtained remarkably rapid injection through holes that had been open to the air for as long as thirty-six hours before liquid was introduced. Other and later workers have also found the exclusion of air unnecessary, for example, Collison, Harlan, and Sweeney (1932).

The attempt to exclude air from the injection hole complicates the actual operation so greatly that for its justification the rate of injection or the thoroughness of permeation would have to be at least doubled. In a number of the earlier experiments leading up to the work described in this paper, and in some done later, comparative tests were carried out sufficiently carefully to reveal differences of this magnitude if they existed, but in none was a definite advantage secured by excluding air.

Influence of soil moisture on injection.

When the soil is comparatively dry the water column in the root of an apple-tree in full foliage is in a state of tension, as is proved by the fact that dyes injected in solution under negligible pressure commonly reach the finest root-tips. The impression was gathered from a number of experiments, that the proportion of dye in the roots to that in the upper part of the tree was smaller with the soil wet than with it dry.

An experiment carried out during the summer of 1932 is of interest in this connexion. A fortnight prior to its start, as a result of drought, the soil and subsoil were comparatively dry down to the underlying rock, subsequently heavy rain fell and the uppermost foot of soil became moist. A large apple-tree in full leaf was injected with dye solution for about 3 days through a hole in its trunk by an early method. The tree was then beheaded and the roots were excavated. A few of the smaller roots which went nearly straight down into the dry soil had become deeply dyed to their tips, in striking contrast to the apparently similar roots that were entirely in the moist top layer of soil which were not dyed at all. The appearance of the main roots was even more striking. A cross-section of each of these near the trunk was partly dyed and partly uncoloured. These and their sub-branches were followed downward until the cross-section of each was either completely coloured or entirely uncoloured. The completely coloured roots were always found to ramify in the dry soil and the uncoloured roots in the wet. It is apparent, therefore, that although this injection was allowed to proceed for three days only the rootlets in dry soil became permeated.

A few further points of interest may be mentioned. The movement of dye

to the very tips of the roots suggests that some of the water in which it was dissolved must have left the root-tip and entered the soil, since water usually moves more freely than the dye dissolved in it. This further suggests that the normal movement of water, and possibly of simple salts dissolved in it, from the soil into the roots may be a reversible process. This movement of substances to the root-tips, and possibly through them, is of some theoretical interest as it suggests experimental and practical possibilities. For example, the thorough injection of roots when the soil is dry suggests that they could be killed by this means, if desired, more efficiently under these conditions than at other times. This increased efficiency might make possible the substitution of *higher* concentrations of substances such as artificial fertilizers, for the highly poisonous substances commonly used for killing trees.

The fact that the movement of injected liquid is influenced by the moisture condition of the soil, coupled with the known effects of climatic conditions on the rate of transpiration, explains to some extent the difficulties in ensuring adequate liquid injection when both the soil and the atmosphere are moist.

Application of whole tree injection.

The injection of whole trees is being used experimentally for purposes similar to those for which branch injections are used, and it is to be preferred to the branch method when a large experimental unit is advantageous. As an example the preliminary work already reported (Hulme, Levy, and Roach, 1937) may be given: the object was to vary the composition of a number of lots of fruit, each consisting of several bushels, to allow of chemical, physiological, and storage tests being carried out on them after picking. In some plantations individual trees may be selected which resemble each other more closely than do the separate branches of any single tree. In these circumstances the whole tree method is obviously preferable to the single branch one. Possible commercial applications of this and similar methods will be considered later (pp. 218-21).

DETERMINATION OF DISTRIBUTION OF INJECTED LIQUIDS

The trustworthiness of all the methods described in this paper depends largely on that of the methods used to trace the distribution of liquid; and this will be true also of any attempts which may be made in the future to modify them for other purposes or to adapt them to other plants. The real value of any injection method, at least from the experimental point of view, may be judged from the extent to which the resulting distribution can be predicted and controlled; and the final test is to ascertain how far prediction coincides with the result of the injection as judged by the distribution of the effects produced by the substance injected—for example, improved foliage colour, increased growth, freedom from disease, &c. In the earlier stages, however, much more rapid methods, such as the use of dyes, have obvious advantages.

The employment of dyes was the earliest of these 'quick' methods used. Next a substance such as a lithium salt was used which is easily detected spectroscopically, or a cyanide, which is readily recognized by chemical means. Lastly the substances suitable for main experiments such as are employed in some of the writer's earlier experiments, may be utilized in such concentration and quantity as to produce slight leaf damage which betrays the distribution of the liquids.

Use of Dyes

Dyes have the obvious advantage of easy and almost immediate visibility. As shown already some idea of the type of distribution resulting from a leaf injection with a dye may be obtained in a quarter of an hour, and the distribution can be mapped in an hour. When main branches or whole trees are injected with dyes the distribution may be seen readily after a few hours if injection is rapid. These remarks, however, apply only to certain dyes.

Reference has already been made to the work of Goppelsroeder (1889, 1901). He found that those dyes which travelled most rapidly up strips of blotting paper dipped into a solution also ascended most readily leafy shoots, the cut ends of which were placed in the dyes. Another method used by him for comparing dyes consisted in dipping a paper strip into a mixture of two dyes. For most mixtures the two colours were shown combined at the bottom of the paper, but above it the colour changed suddenly to that of the dye which travelled the faster. This method of mixing dyes in pairs was used by the writer to compare a number of dyes both with strips of blotting-paper and also on plant material. With plants two methods were used: in one the cut ends of shoots were immersed in the mixture, and in the other the mixture was dropped into shallow holes bored in the trunk. The single dye solutions were also dropped into similar shallow holes. All these methods gave substantially the same results which were in harmony with those obtained with the same dyes when used for injection work. In the following table, twenty-three dyes are placed in the order determined by these tests, beginning with those which travelled most freely and ending with those which hardly travelled at all.

TABLE II

1. Acid Fuchsin	9. Azo Geranine	17. Methyl Orange
2. Light Green	10. Lissamine Fast Red	18. Methyl Violet
3. Lissamine Fast Yellow	11. Coomassie Yellow	19. Neutral Red
4. Patent Blue	12. Solway Blue	20. Congo Red
5. Ponceau Red	13. Alkali Blue	21. Crystal Violet
6. Water Blue	14. Erythrosin A Eosin	22. Fuchsin
7. Tartrazine	15. Erythrosin B Eosin	23. Bismarck Brown
8. Amaranth	16. Methylene Blue	

Case (1938) has found orange G a satisfactory red dye. Methylene blue, which was found practically useless by the present writer for apples and other trees, has been used successfully by other workers for other plants. The

dyes found most suitable in the present work were acid fuchsin (red), light green, and patent blue. The last two were first used for this purpose by Harvey (1930), and the second of these he listed under its synonym, 'brilliant blue'. All three dyes are in 0·1 per cent. solution nearly harmless to living plant cells, the least harmful being patent blue, which is probably the most generally useful for injection work. Only highly purified dyes should be used, otherwise there is a risk of the wood vessels becoming choked by impurities, some of which seem to become precipitated slowly from solution on standing. The first two dyes, as supplied for microscope work, and the purest grade of patent blue, as supplied by chemical firms, all give satisfactory results.

In a number of cases the distributions first determined with these three dyes have since been verified by the results obtained with nutrient solutions. These include results from the interveinal method on the apple (see p. 176), and the broad bean (by H. Hill, details to be published shortly), from the leaf-stalk method on the apple (Roach and Levy, 1937), from the shoot-tip method on the apple (this will be mentioned again shortly) and cherry (Roach and Levy, 1937), and from the individual branch method on the pear (Srivastava and Roach, 1937), and the apple (Sen, 1937).

The use of nutrient solutions in such concentration and amount as to cause slight leaf damage has given successful results on a number of occasions. Its limitations as a method are suggested by an experiment already described on pp. 210–11 in which part of the tree was permeated, not sufficiently so as to cause damage, but sufficiently for subsequent growth to be increased materially.

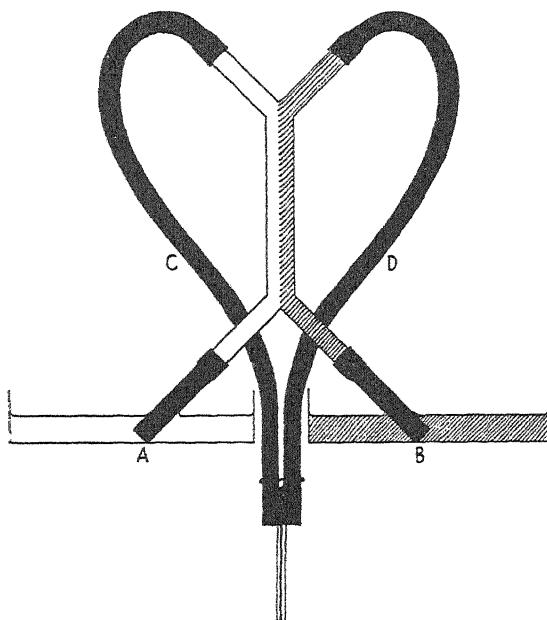
An early experiment already mentioned on p. 194 (Roach, 1934a) serves as an example of roughly the same degree of permeation recognized by the damage caused by sodium thiosulphate injected through the shoot-tip, by dye injection in a similar manner, by the apparent resistance to mildew and by the increased growth following the injection of the sodium thiosulphate.

Suitable dyes may therefore be used with considerable confidence for showing the distribution likely to result from any particular type of injection. The small differences in the extent of permeation due to variation in the plant and in the injected substances themselves are easily allowed for in practice.

LOCALIZATION OF EFFECTS OF INJECTION

The value of all the methods for injecting parts of plants, in fact all those described in this paper except that for injecting whole trees, depends on the conducting channels for various parts of the plant being to a certain extent independent. Auchter (1923) concluded from his own experiments and from those of others that the mineral nutrients absorbed by the roots on one side of a plant are in large measure used by the leaves in direct connexion with those roots, and that there is apparently very little transfer of such nutrients from one side of the plant to the other. As was shown in an early paper by

the present writer (Roach, 1934), if a tubular glass contrivance such as that shown in Text-fig. 34 be made and supposed to represent a tree with its roots, stem, and branches, and if the two lower 'roots' A and B be dipped into two differently coloured liquids of the same density, then when uniform



TEXT-FIG. 34. Simple model of root and shoot system to show two streams flowing through the system without mixing.

suction is applied to each 'branch' C and D through the black rubber tubing shown, the liquids will travel up the central tube in parallel columns which do not mix. The columns pass into the 'branches' at the upper fork, still without mixing. The liquid in each 'branch' may be all of one colour or be of two colours, side by side, without lateral mixing. The pull of the leaves is represented by that of the curved rubber tubes acting as syphons, from the ends of which the liquids are allowed to discharge at uniform rates down a piece of cotton to prevent the slight irregularity of movement if the liquid is allowed to drop from the ends of the tubes. Freedom from vibration is essential for success of the experiment. With this apparatus unequal soil moisture in the two roots and unequal transpiration in the two branches may be imitated by constructing a 'root' or 'branch' rubber tube by means of a screw clip. There is no mechanism in a tree tending to mix these streams, on the contrary the structure of plants tends to prevent such mixing.

Thus in numerous early experiments leafy trees of various sizes were lifted and the cut end of a single root of each was immersed under one dye solution

and the remaining roots were immersed in another dye solution. In each experiment the dye could be traced up the strands in the main stem which were in direct connexion with the single root and into the leaves at their other end. There was never any mixing of the dyes. Even when only a single root was immersed in a dye, the remaining roots being allowed to dry, the dye ascended the strands in direct connexion with it for a distance of many centimetres before any movement into contiguous strands could be detected. A similar result was obtained when the cut end of a branch was immersed; the dye descended the strands in direct connexion with it for a similar distance before invading neighbouring strands and entering the leaves in connexion with them.

Justification for the employment of plant injection methods lies in the results already obtained with them. These include the following: (i) The effects of interveinal injections have been confined to the injected areas until the leaves have fallen naturally in the autumn. (ii) The injection of iron sulphate in the autumn of 1935 into shoot-tips of chlorotic cherry-trees resulted in the turning green of a number of leaves at the end of injected shoots; these remained green not only during the summer of 1936 but the same shoots were green in the autumn of 1937, still in striking contrast with the rest of the tree. (iii) The effects of injections of branches have remained confined to those branches for a whole season. How long the effects of injection remain localized is still unknown, but the above facts open up a sufficiently wide field of usefulness.

'INJECTION' WITH SOLIDS

In the course of the present work solid substances have been used for injection on a few occasions only. Solutions must obviously be used for all the leaf-injection methods, and their more rapid distribution make them more convenient for working out general principles. Mention has already been made of some of the results obtained by other workers with solids, particularly when applied to whole trees and sometimes even on a large commercial scale. In view of the many factors already noted which tend to influence the injection and distribution of the liquid used, it has seemed unwise up to now to attempt any comparison between the solid and liquid methods in circumstances in which both are applicable; but such comparisons should soon be possible. Only a few tentative remarks can be made at present. Leaf-injection methods, the quickest for diagnostic purposes, are feasible only with liquids. Solids cannot be used for the shoot tip method and hardly for the branch methods, at least with small branches. For larger branches and whole trees, the response to solid injection appears to be slower than that to liquid injection; circumferential movement seems less from a solid than from a liquid injection and consequently a larger number of holes is necessary for the former. Nevertheless, the fact that no liquid is necessary for a solid injection is definitely in its favour for commercial use. Further work is

obviously necessary before the best methods for all purposes can be decided on and a number of possible modifications remain to be tested.

GENERAL APPLICATION OF PLANT-INJECTION METHODS

The results stated in this paper are by themselves enough to suggest that each of the common nutrients produces the same effect on a plant whether it is injected artificially or is absorbed by the roots in the ordinary way. Consequently the 'artificiality' of the injection method of supplying the test nutrients does not invalidate its use in the diagnosis of mineral deficiencies and in physiological studies generally.

By one of the methods described a single interveinal area of the leaf of certain plants may be injected with a test liquid, which becomes so distributed that the treated area is separated from the untreated areas on each side of it by a secondary vein. This separation is so sharp that the slightest change in colour or texture of the treated area is easily detected, permitting mineral deficiency to be diagnosed in a week, or even less. Lal, working on the soya bean, has recently obtained a response to iron two days after injection. Further, the occurrence of single interveinal areas, healthier than the rest of the leaf, is so uncommon in untreated leaves that the probabilities are a thousand to one against the response shown by a single treated area being due to chance; and if the experiment is carried out in duplicate the odds become a million to one, a degree of certainty rarely achieved in experimental work on plants.

An equally rapid diagnosis may be obtained, with the same degree of freedom from interference from natural variation, by the leaf-stalk method, which, when applied to apples and many other plants, results in a number of selected leaves becoming injected on one side of their midrib but remaining untreated on the other. These two methods can be carried out quickly and entail only negligible interference with the tree or plant; consequently they seem suited both for experimental trees and for those growing in commercial plantations. They appear to hold out the possibility of diagnosing not only gross mineral deficiency but also the early stages of such deficiencies. It should therefore be possible to avoid the worst manifestations and adverse results of mineral deficiency by correcting the tendency at an early stage, and also to maintain trees in a much higher condition of health than is possible at present.

As already noted, it took no more than two hours to carry out the injections which demonstrated the widespread occurrence of incipient chlorosis of the iron shortage type in the plantations of the East Malling Research Station (Roach and Levy, 1937). This work was due to the suspicion entertained for some years that a lowered content of organic matter in the soil was leading to a diminished availability of iron and possibly of other elements. Probably this applies to wide areas of fruit cultivation. The remedy would seem to be a change in cultural practice to increase the soil organic matter, but while this somewhat slow-acting cure is being employed the more seriously affected

trees can be cured rapidly by injection. Injection also seems to be the only cure which will act quickly enough to save considerable numbers of chlorotic fruit trees growing in excessively calcareous soils. The method is being applied for both purposes on a commercial scale.

The soils of wide areas of the world have become seriously impoverished in organic matter and as a consequence the mineral salts previously held by the organic matter, have been leached away. It is doubtful whether the mere replacement of the organic matter alone will ever result in the conversion of enough of the salts from the solid soil particles to a more available form, and it is still more doubtful whether this will occur in a reasonable time. The problem will have to be faced of first determining the minerals that are deficient in quantity and then replacing them by temporary or permanent measures. Injection is probably the simplest diagnostic method and it is worth considering as a curative method in exceptional circumstances.

The shoot-tip, the branch, and the whole tree injection methods are neither as sensitive nor as rapid as the leaf methods, but they may be used for studying gross effects on growth response and on yield and quality of fruit. The value of all these methods in general fruit advisory work is being tested by the Advisory Officers in Commercial Fruit Growing, Messrs. W. G. Kent, C. R. Thompson, and J. B. Duggan, in co-operation with the writer, throughout the county of Kent. The methods are also being tested on a variety of crops by other workers in this country and in many overseas areas.

The drift soils on which fruit is largely grown in the south-eastern counties of England vary so markedly, even within short distances; and their manurial treatment, which has often been very heavy, has varied so greatly from place to place, that the more detailed manurial problems can be solved only by experiments carried out in the commercial plantation itself. The degree of randomized replication necessary in manurial experiments devised to deal with such problems, and their cost and inconvenience, make them inapplicable to commercial plantations. This, therefore, appears to be an important field of work to which injection methods may well be suited.

For these, and other types of problems to be mentioned later, injection methods seem likely to have a theoretical, and later a practical, advantage over even the best-planned manurial experiment. It is well known that when a substance, such as a copper salt, is applied to soil, the greater part of the copper, even 99 per cent., is precipitated or absorbed into the solid part of the soil, while there is liberated into the soil solution an equivalent amount of a number of other elements. The observed effect on the plant by its application to the soil may be due either to the 1 per cent. of the copper salt left in solution or to any of the substances liberated, or to both.

Another instance of the practical importance of copper salts has recently been discovered by Teakle (1937) and Dunne (1938) in Australia by the use of injection methods. It was found that a serious dieback disease could be cured by injecting a copper salt, and later soil application was found to be effective.

The work of Allison, Bryan, and Hunter (1927) has shown that copper, manganese, or nickel can each be of great benefit to crops on an exceptional type of peat soil. There is increasing evidence that the functions of most, if not all, of the elements are quite specific and that one element cannot replace another. It is tempting to wonder, therefore, whether the similar, immediate, practical value of these three elements is not really due, at least in part, to base exchange of the type described above. If this be so the determination of which elements are actually used by the plant is likely to be one of the utmost practical importance in the not distant future. Such a problem can be solved quickly and simply by injection methods.

There is a far-reaching problem of this type of immediate practical importance in fruit growing. Fruit trees, in general, require large quantities of potassium, and it is a common experience that large quantities must be added to the soil before any appears to reach the tree, and some years elapse before there is any improvement in its condition. In such circumstances the injection of all trees in a plantation to make sure of attaining an immediate response may well be the most practical solution. At the same time, or later, the systematic treatment of the soil can be taken in hand.

The cause and cure for a disease of apples of great economic importance, especially in Canada and New Zealand, have recently been discovered by injection methods. Atkinson (1935) in New Zealand, McLarty (1936) in British Columbia, and Young and Bailey (1936) in New Brunswick, working independently, each found as a result of injecting a number of substances into apple-trees that boron compounds alone cured the trouble; and a little later Jamalainen (1936) confirmed this discovery by experiments carried out in Finland. These workers have determined suitable dressings for application to the soil and there is consequently a commercially practicable cure for the trouble on most soils. Calcareous soils still, however, present difficulties (Hill, personal communication), but until these are overcome the disease may perhaps be cured commercially by injection. The disease has been known by various names, e.g. corky core, drought spot, &c., and shows a number of widely different symptoms which have been confused with others similar in appearance, but evidently due to a different cause. It is now possible to group together all those symptoms cured by boron (Hill, 1937). Having verified by injection the fact that the remainder are not thus curable (Levy and Roach, 1937) it is proposed to seek for cures for these by similar methods, but using different compounds, guidance as to what substances to try being obtained by chemical and spectrographic analysis of healthy and affected plants.

In experiments already described (p. 211) the injection of apple-trees with a solution containing dipotassium hydrogen phosphate and urea was followed by a marked increase in vigour; the treated trees were free from two insect pests, leaf hoppers (*Jassidae*), and red spider (*Oligonychus ulmi* Koch), in marked contrast to the untreated trees which were heavily infested; and as

already stated some unpublished results obtained by H. Wormald and the writer suggest that silver leaf may also be controlled by the injection of nutrient salts or other substances. Similar methods offer a comparatively simple means of determining how far insect and fungus diseases can be controlled by adjusting the nutrition of the host.

Another problem may be mentioned here since it led to the development of these injection methods and that is the stock-scion relationship. The effect of stock on scion is of great physiological interest and of marked horticultural importance. One of the ways by which the stock may act is by controlling the supply of mineral elements to the scion (Roach, 1931). In investigating the effect on the scion of the numerous mineral elements (almost twenty) transmitted by the stock the injection method has obviously many advantages.

Since fruit may be injected while still on the tree or even after it has been picked its content of various substances may be altered and a study made of the effect of such alteration on the disease resistance, keeping quality and general physiology of the fruit.

SUMMARY

1. A review is given of the history of plant injection, going back to the twelfth century.
2. Methods are described for injecting individual parts of a plant each with a different liquid, using the untreated parts for comparison.
3. The methods may be used for several physiological purposes and especially for the diagnosis of mineral deficiencies.
4. Such diagnosis may be carried out in as short a time as four days.
5. Some of these methods have a high degree of statistical reliability.
6. Single interveinal areas of certain plants (e.g. apple, pear, strawberry, &c.) may be injected so that the permeated area is sharply separated by secondary veins from the untreated area on either side. This method is not applicable to the peach and the tomato.
7. A leaf after its apical quarter has been amputated may be injected while still attached to the tree by immersing the cut edge.
8. Similar treatment of the leaflet of a compound leaf in some plants (e.g. the strawberry) results in the permeation of one side of the adjacent leaflet, while the other side is not permeated.
9. Injection, through a leaf-stalk left attached to the stem after the blade has been removed, results in the permeation of whole leaves and parts of leaves above and below the injection point, the 'injection pattern' depending on the phyllotaxis and the vascular anatomy of the stem. In some leaves the midrib forms a sharp division between permeated and unpermeated blade tissues.
10. Injection through a shoot-tip results in the permeation of a number of the terminal leaves.

11. Branches may be injected independently of the rest of the tree through a suitably placed hole passing diametrically through the branch.
12. The individual branches of certain trees may be injected each with a different liquid so that the corresponding roots are also permeated.
13. Whole trees and other plants may be injected through a single diametrical hole, large ones through two or more radial holes.
14. Uniform permeation of all the branches depends on the proper placing of the hole or holes in relation to the branches.
15. Examples are given of physiological and practical applications of these methods.

ACKNOWLEDGEMENTS

Much help has been acknowledged at various places in this paper. The writer wishes to thank Mr. D. Akenhead, Professor V. H. Blackman, and Dr. G. H. Pethybridge for many valuable suggestions concerning the presentation of this paper and Mr. D. Akenhead for seeing it through the press during the writer's absence in South Africa.

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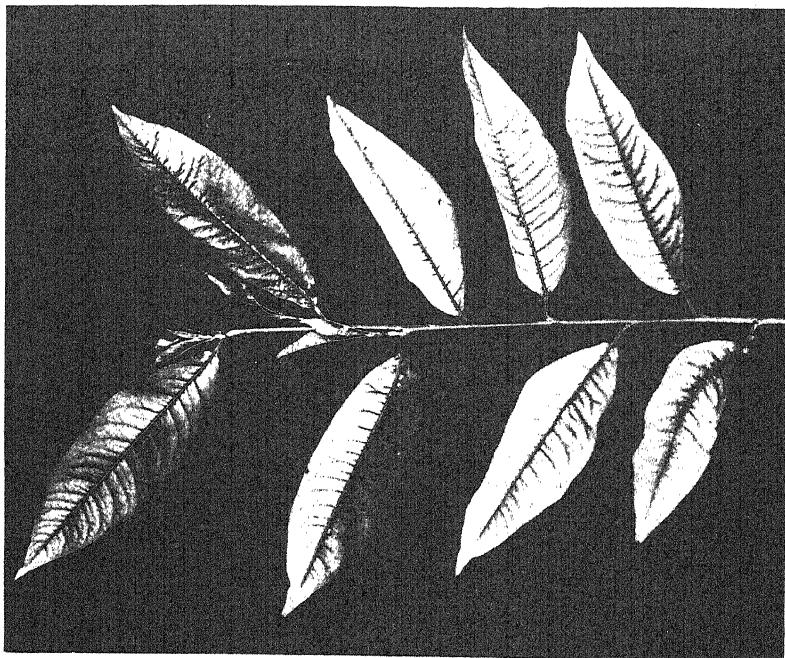
EXPLANATION OF PLATE IV

Illustrating Dr. Roach's paper on 'Plant Injection as a Physiological Method.'

Fig. 1. The effect of injecting urea into a young broad bean leaf through an incision just below the arrows, which indicate the limits of permeation of the urea. (Photograph by Miss K. E. Cornford.)

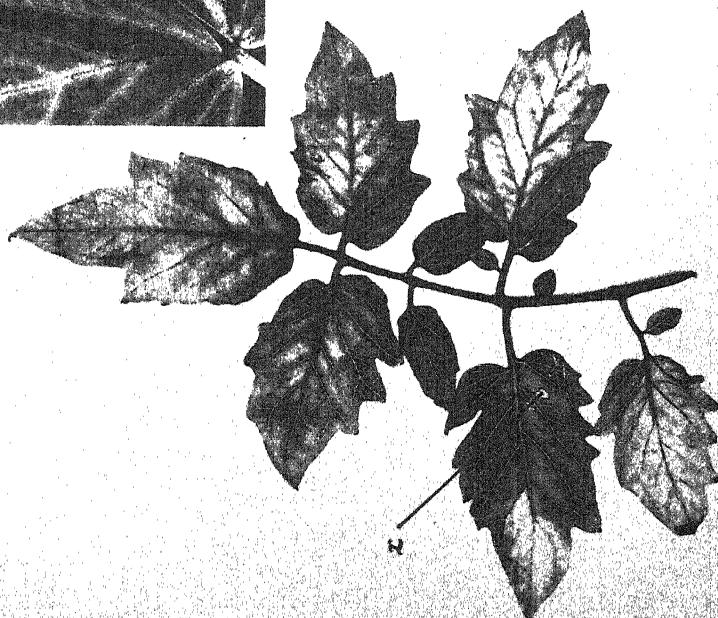
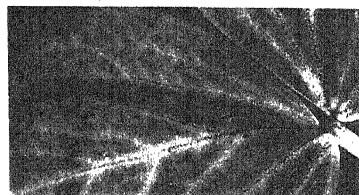
Fig. 2. The effect of injecting sodium borate through the incision at x into a young tomato leaf. Note how widespread is the effect compared with that of urea in Fig. 1. (Photograph by Miss K. E. Cornford.)

Fig. 3. Effect of the injection of 0·1 per cent. ferric chloride into a shoot of a chlorotic peach tree. The two terminal leaves and half of the third have regained their normal colour.



1

3



2

The Effect of Puncturing Individual Latex Tubes of *Euphorbia Wulfenii*

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THE object of this work was to observe the effects of puncturing single latex tubes *in situ* in the living tissues of a laticiferous plant. *Euphorbia Wulfenii* was selected for this purpose because the latex tubes of its stem are large, averaging 30μ in diameter, and exude copious quantities of latex. Moreover, they are confined to the phloem and run almost entirely in a longitudinal direction with only occasional bifurcations.

On taking a shoot from the plant the cut end was immediately plunged into alcohol to coagulate the issuing latex and so prevent excessive bleeding. The end was then washed and transferred to water until required for use. Portions of tissue for observation were removed from the stem by making two horizontal cuts down to the wood about 5 cm. apart, and then two longitudinal ones about $\frac{1}{2}$ cm. apart so as to enclose a rectangular strip of tissue which could readily be peeled away from the central woody core. Owing to the arrangement of the latex tubes the longitudinal cuts damaged only those tubes at the edges of the strip, while the horizontal ones severed practically all of them. Before making the latter cuts, therefore, the razor blade was heated sufficiently to coagulate the latex at either end of the strip and so block all the ends of the tubes.

When the strip was removed from the stem it was at once plunged into liquid paraffin to prevent evaporation from the exposed surfaces and to wash away drops of exuded latex. It was then blotted and pressed, epidermis downwards, on to a block of plasticine previously fixed on a glass slide. The exposed cambial surface was covered with liquid paraffin to prevent superficial glare when examining by reflected light, and all subsequent operations were performed within this medium. The slide was then clamped in the moving stage of a microscope fitted with a Leitz $\frac{1}{4}$ -inch 'Ultropak' illuminating objective and a $\times 6$ eyepiece.

The latex tubes of the inner layers of the phloem could be seen through the cambial cells as white streaks, and by traversing with the moving stage could generally be traced for the whole length of the strip. Sometimes, however, they would vanish into the deeper layers of the tissue or terminate in

a blunt end, while very occasionally they would make their way between the cambial cells and appear for a short distance at the surface of the tissue. Only in the latter cases was it possible to make any useful observations upon the tubes, which were then seen to be thin-walled and entirely filled with a clear bluish-white opalescent fluid which on coagulation changed to an opaque clotted white mass. Even when coagulated the latex generally more or less completely filled the tube, although occasionally it was observed then to take the curious form of a spirally twisted ribbon.

Puncturing of these superficially appearing portions of the tubes was effected by means of a simple type of micromanipulator designed by Pyke (1939), using needles made of finely drawn-out glass rod. Before performing this operation it was generally necessary to trim away some of the adjoining tissue in order to give free play to the needle, which was manipulated so as to approach the tube at right angles. When a point was driven into and then withdrawn from an undamaged tube containing fluid latex a droplet of the latter, of diameter from one to three times that of the tube (i.e. about 0.0001 cu. mm.), was immediately extruded, without any measurable contraction of the tube. If the strip was rendered fully turgid by previously soaking in boiled distilled water before covering with paraffin, then a much larger drop was yielded, but still without visible decrease in tube diameter. If any contraction occurred it must have been less than 5μ .

The drop of latex either gradually dispersed in the liquid paraffin, its original sharply defined edge becoming more and more diffuse until it was eventually quite invisible, or else it exhibited a partial coagulation. The stages of this latter process could readily be examined by repeatedly touching the surface of the droplet with the glass needle. At first the surface-tension attraction between liquid and glass would cause an immediate formation of contact menisci, but as the viscosity increased this reaction occurred progressively more slowly, and on drawing the needle away from the surface, strands of latex would partially adhere to it instead of the previous rapid adjustment to the convex surface of the droplet. When the surface had finally coagulated to form a skin, then contact with the needle produced an indentation.

On repuncturing the tube after a period of about one minute the extrusion of a fresh droplet of latex was almost always recorded. Its size varied with the time interval allowed, but it was never so large as the initially formed one. A closure of the original wound followed by a partial turgor recovery within the tube is thus indicated. A third puncture after another time interval would generally yield another smaller droplet and, in fact, the process could be repeated either until the latex in the tube began to coagulate or until entry of the immersing liquid paraffin became evident. In this connexion it was observed that repeated puncturing in one place often caused a localized coagulation inside the tube, so it is possible that the wound-closure noted above is produced by latex particles that have coagulated around the hole. If too large a hole was made, however, it did not heal itself and the

paraffin entered by capillarity, exhibiting a convex meniscus of contact with the latex and gradually displacing the latter from the tube.

On one occasion a series of three or four latex droplets of progressively diminishing size was observed to exude at about two-minute intervals from the original puncture without the application of any additional needle-thrusts. These droplets did not completely coalesce owing to the partial coagulation of the first. It would appear, in this instance, that the original puncture was repeatedly opened up again each time the turgor pressure in the tube reached a value high enough to overcome the resistance of the closing obstruction.

Whether this wound closure is due to coagulated latex as suggested above, or to some property of the wall, is at present unknown. Manipulation with a blunt needle certainly indicates that the tube walls have the properties of an elastic membrane, but they are so thin that attempts to demonstrate the presence of a protoplasmic lining by plasmolysis have so far failed owing to the difficulty of observing them under reflected light.

SUMMARY

A method is described for puncturing single latex tubes *in situ* in the living tissues of a laticiferous plant.

The results of this operation are recorded for *Euphorbia Wulfenii*, and it is shown that the plant possesses a mechanism for closing the wound so produced, so that rapid turgor recovery of the tube occurs.

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On the Nature of the Blocking of the Laticiferous System at the Leaf-base of *Hevea brasiliensis*

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With one Figure in the Text

IT was originally noted by Parkin (1900), and later mentioned by Lee (1911) and confirmed by Bobiloff (1918), that when the petiole base of a mature leaf of *Hevea brasiliensis* was cut through in the region of the separation layer, bleeding of latex did not occur from either the petiole or stem side of the cut, although when a young leaf was severed in the same region free bleeding took place from both exposed surfaces. This observation was repeatedly confirmed by the present author during the course of physiological experiments upon this plant. Since a cut on either side of the separation layer of the mature leaf would produce bleeding, it was suggested by Parkin that a blockage was produced in this region of the laticiferous system, possibly owing to pressure of calcium oxalate crystals against the tubes. The present investigation was undertaken principally to determine the cause of this phenomenon.

Methods employed

The paraffin-wax microtome technique was soon abandoned as it was found impossible to stain the latex differentially after passage of the sections through a wax solvent. A solution of gentian violet in glycerin jelly would often give a deep stain to the latex when sections were mounted in this medium, but the reaction was very variable and moreover only occurred when the sections were mounted near the edge of the coverslip. The precise reason for this was not determined, although it was shown not to be due to atmospheric oxidation. Iodine green, also, after prolonged contact would sometimes impart a deep purple colour to the latex; while methyl green would sometimes stain it blue.

The common fat stains, however, all yielded a rapid and certain reaction, especially if the sections were previously treated with eau de Javelle to remove the tannins which are always present in considerable quantities in most parts of this plant, and which greatly impede the staining. Chlorophyll solution, alkannin tincture, Scharlach red, and Sudan III were all found useful, and the latter was selected as giving the deepest stain to latex.

On cutting portions of tissue from the plant they were at once plunged into 95 per cent. alcohol in order to coagulate the latex quickly at the cut surfaces and so prevent its loss. They were then either left in this reagent, or else, in the case of the less lignified tissues, transferred to 70 per cent. alcohol with or without the addition of 2 per cent. formalin. The material was not used until, after the duration of several days, the chlorophyll had all dissolved out. Much longer periods of immersion in alcohol, however, reduced the staining capacity of the latex.

Where serial sections were required the freezing microtome was used. For this purpose it was necessary to replace the alcohol in the material by water. This was done by transference to distilled water and distillation of the alcohol under a suction pump. The pieces of tissue, which had previously been trimmed down to the smallest convenient size, were then frozen in mucilage of tragacanth on the microtome stage and sections cut at 50 or 100 μ thick. It was found that the microtome would not cut good sections unless all the air, present in the pith cells of the lignified stems, was removed; for this reason it was not generally possible to cut fresh stem material on this microtome. The alcohol fixative, however, was found readily to replace the air, although if this alcohol was not subsequently replaced completely by water the sections would not cut cleanly owing to its lowering of the freezing-point.

As the sections were cut they were transferred to water in a series of watch-glasses to wash away the gum, maintaining the correct serial order. They were then transferred to eau de Javelle for five or ten minutes until completely bleached. After again washing in water they were stained for half an hour or longer in a 1 per cent. alcoholic solution of Sudan III diluted with an equal volume of glycerin. They were then given a brief wash in 10 per cent. alcohol and transferred to dilute glycerin, in which medium they were arranged in their correct order on slides and finally mounted in glycerin jelly.

General observations upon the laticiferous system

By means of the above technique the latex and the cuticle were stained deep orange, the rest of the tissues remaining almost colourless. Storage fats in the cells of course also took up the stain, but these are abundant only in the seed. The latex tubes are very thin walled, and in the mature plant apparently without transverse septa. Those that were not full of latex, however, did not appear collapsed, and the residual portions of coagulated latex in them had rounded convex ends, the smaller fragments being roughly spherical and not generally adhering to the walls. It would thus appear that before coagulation the latex did not wet the inside walls of the tubes. The coagulated latex was densely granular in appearance and sometimes contained clear unstained globules: apart from these, however, all parts of it were evenly stained the same colour.

In all parts of the plant the latex tubes are mainly confined to the region of the phloem, where they form a complex branched system ramifying

between the other cells and frequently running in close contact with the sieve tubes. A few latex tubes are also always present branching in a similar manner between the cells of the cortex, connexion between the phloem and cortical tubes being maintained in the stem by branch tubes passing through the intervening pericyclic fibres. A few tubes are to be seen in the medulla, running mainly in a longitudinal direction: these make connexion with the phloem system only through the parenchymatous regions where leaf traces leave the stem. Latex tubes were never observed in the xylem nor in the numerous starch-filled medullary rays.

In the leaves, also, the tubes are principally confined to the phloem, but as Haberlandt (1928) has recorded for other laticiferous plants, numerous branches pass out from the main system and make their way up between and in close contact with the cells of the palisade layer and end blindly at the epidermis. Some actually run for a short distance between the epidermis and palisade before finally terminating. A few branch tubes also pass downwards in a similar manner to the lower epidermis upon which the stomata are borne.

ARRANGEMENT AT THE LEAF-BASE

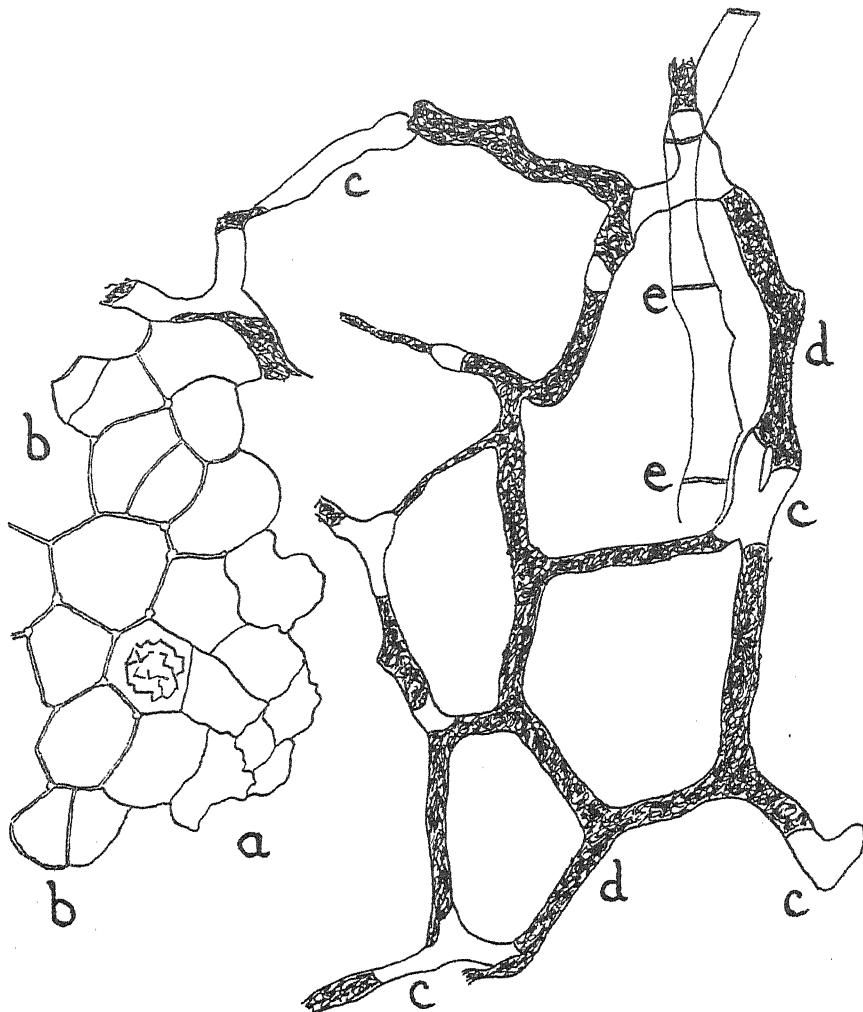
Some eighteen vascular leaf traces leave the stem at each node and pass out through a gap in the cylinder of pericycle fibres into the petiole. The exact manner in which they bifurcate and anastomose has been examined by means of serial sections and by blocks of cleared nodal tissue stained with fuchsin, but need not be described in detail here. The latex tubes pass out into the petiole principally in the phloems of these traces, but there are generally a number of them branching in the cortex and medulla as well.

The separation layer of the mature leaf consists of cells with very thin walls which, prior to abscission, generally have a wrinkled appearance (see *a* in figure). On the leaf side of this layer the cells undergo divisions in a plane parallel to it and become slightly lignified (*b*). On the stem side of the separation layer dense deposition of calcium oxalate crystals occurs, while between this region of crystal formation and the main stem a number of scattered stone cells are always to be seen.

In the region of the separation layer the latex tubes were observed to be completely occluded in many places by a highly refractive, unstained, transparent substance (*c*), in contact with which the adjacent latex (*d*) often formed marked concave menisci. Testing with aniline blue, and also with corallin soda, indicated this substance to be callose.

DOUBLE-STAINING TECHNIQUE

The latex and callose could be differentially stained to give permanent preparations by means of Sudan III and aniline blue, but since the latter dye is readily bleached by eau de Javelle, it was found necessary to remove this from the sections by washing for half an hour or more in 1 per cent.



TEXT-FIG. Tangential longitudinal section of mature leaf-base of *Hevea brasiliensis* through phloem region of a vascular bundle. *a*. Thin-walled, wrinkled cells of separation layer. *b*. Lignified parenchyma at petiole base. *c*. Portions of latex tubes occluded by callose. *d*. Portions of latex tubes containing coagulated and stained latex. *e*. Sieve tube with callosic sieve plates. (Parenchyma between the latex tubes is omitted for clarity.)

hydro-chloric or acetic acid after the bleaching process, subsequently neutralizing the acid by washing in dilute ammonia. Hydrochloric acid was found to remove the residual eau de Javelle the most effectively, but it generally caused some destruction of the hypodermal tissues, the cells separating from each other in this region and the epidermis curling back from them.

After washing out the ammonia the sections were stained first in Sudan

III as described above and then for half an hour or more in dilute aqueous aniline blue. They were then rinsed in water and left in dilute glycerin until only the callose of the latex tubes and of the sieve plates (Text-fig. e) retained the bright blue aniline stain; they were finally mounted in glycerin jelly.

TIME AND PLACE OF CALLOSE DEPOSITION

The deposition of callose in the latex tubes at the separation layer was found to occur at the time of secondary thickening of the petioles and of the stem on which they are borne. In no case was it found in the immature leaves on the young un lignified shoots. By the time the leaves had reached maturity and the three laminules acquired their final green colour and erect posture the callose formation had reached its maximum, and the isolation of the laticiferous system of the leaf from that of the stem was complete.

At the bases of the three petiolules at the distal end of the petiole there are secondary abscission regions. These separation layers are anatomically similar to the main one, but are functional only if the laminules die prematurely. Many of the latex tubes in these regions also become occluded by plugs of callose after the leaf has reached full maturity, but in this case the process appears to be incomplete, for, as Parkin and Bobilioff noted, a cut here always produces bleeding from both exposed surfaces.

The physiological causes and functions of callose formation in the latex tubes are at present unknown, but it is of interest to compare this deposition with that which occurs upon the sieve plates of sieve tubes and, more especially, with that found by Oliver (1887) occluding the trumpet hyphae of Laminarieae.

As Bobilioff points out, this occlusion of the tubes lends no support to Haberlandt's theory of the translocatory function of latex tubes, in spite of the observed anatomical relationship between them and the palisade. It also suggests that regeneration of latex after tapping does not take place in the leaves.

SUMMARY

A method is described for the anatomical examination of laticiferous systems.

The arrangement of the latex tubes in *Hevea brasiliensis* is recorded, and the occlusion of the system at the mature leaf-bases of this plant is shown to be due to the deposition of plugs of callose in the tubes.

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Latex Outflow and Water Uptake in the Leaf of *Ficus elastica*

BY

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With one Figure in the Text

THE progressive dilution of latex flowing out from a cut was noted by Arisz (1928), Zimmermann (1927), and Frey-Wyssling (1932), and suggested to these investigators that local reduction of pressure in the latex tubes at bleeding caused an increase in their suction pressure, so that water was withdrawn from the surrounding tissues whose suction pressure was thus simultaneously raised. This process was also suggested by experiments performed by the author (1939) in which single living latex tubes exhibited a rapid recovery of turgor after the cessation of bleeding induced by puncturing.

EXPERIMENTAL METHOD

An attempt was made to examine more directly the effects of bleeding upon the suction pressure of the tissues, and leaves of *Ficus elastica* were selected for experimentation. By steaming a short length of the petiole to produce local coagulation the leaf could be cut from the plant without any latex flow resulting. It was then fitted into a potometer, and after the water uptake rate had reached a steady value the apex of the lamina was cut off with scissors about 1 inch from the tip. Subsequent bleedings during the experiment were induced after various time-intervals by cutting further strips, about a quarter of an inch wide, from the top edge.

The figure represents an example selected from a dozen such experiments. Uptake rate (mg. per min.) is plotted against time, and the arrows indicate the times of cutting. It will be observed that at each bleeding the uptake rate rises, in about one minute, to a maximum and then falls at a decreasing rate until, after about twenty minutes, a steady value is again reached. This steady value represents the transpiration rate and exhibits a gradual decrease from 1.9 to 1.2 mg. per minute during the first part of the experiment, followed by a rise to 2.9 mg. per minute on the following day, these changes probably being due to stomatal variation. In some experiments the transpiration rate was kept almost at zero by vaselining the lamina.

In the experiment presented the third cut was made before a steady uptake

rate had been re-established, but the curve rises to a secondary maximum and then falls as before. From the figure it appears that the difference between the transpiration rate and the maximum uptake rate attained, ranging from 16.4 to 0.5 mg. per minute, is progressively less at each of the five cuttings. This phenomenon was observed in all the experiments, although there was considerable variation in the value of the first maximum attained in each case.

Leaves that were cut from the plant without first steaming the petiole, having already lost a considerable quantity of latex, showed a rise in uptake rate of the order of only 0.5 mg. per minute at the first experimental bleeding. A few leaves were cut from the plant without previous steaming, and kept in a warm humid atmosphere with petioles under wet sand for a week in order to see if there was any evidence of latex regeneration under these conditions; but the subsequent first experimental bleeding was still accompanied only by this same low order of increase in uptake rate.

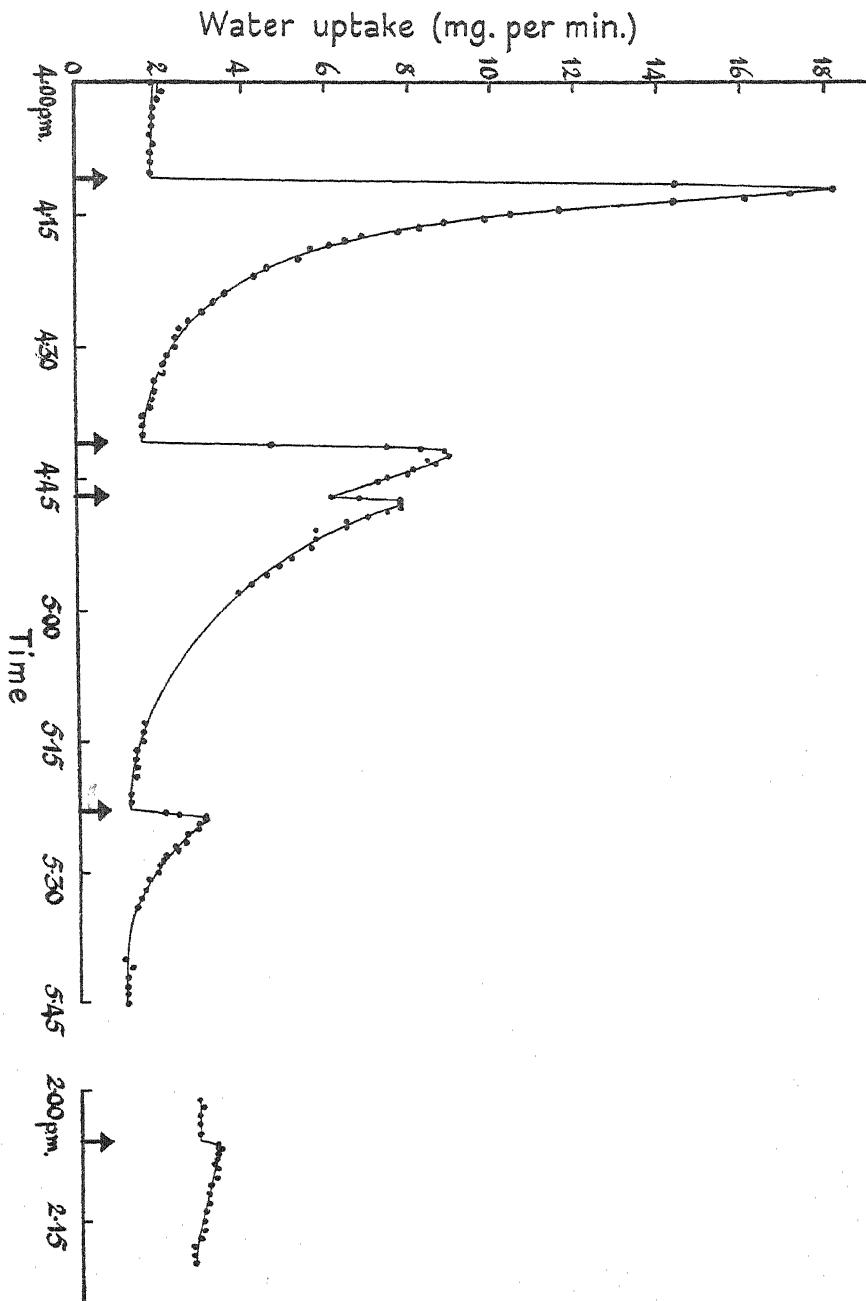
The rate of latex outflow is always maximal immediately after the cut is made and then falls off at a decreasing rate, finally ceasing after about fifteen minutes. This observation confirms unpublished quantitative experiments performed on leaves of this plant by Mr. Pyke in this Institute, in which the course of the latex outflow was followed by a weighing method.

The effect of artificially reducing and prolonging the bleeding period was examined by making the cuts at the leaf-tip under alcohol and dilute ammonia solution respectively. In the first case the latex coagulated within one second and prevented any further outflow. The water uptake curve, however, was of similar form to that in the figure, and showed the usual decrease in the maximum at each cutting. When the tip was cut under ammonia the outflow of latex was observed to continue, at a slow steady rate, for over twenty minutes. This was reflected in the water uptake curve by the fact that it returned from the maximum to a steady value rather higher than it had originally been. On recutting the leaf-tip the uptake rate, however, rose to a maximum again as before.

DISCUSSION

Assuming a constant resistance to water movement from the potometer to the mesophyll, the suction pressure of the latter may be regarded as always proportional to the uptake rate. The initial rapid rise in this suction pressure when the latex tubes are opened may be attributed to one or both of the following causes. Firstly, relaxation of turgor in the tubes may reduce the intercellular pressure due to the mutual pressure of the cells and tubes (Ernest, 1934), and so raise the suction pressure of the mesophyll cells; secondly, the rise in suction pressure of the tubes, necessarily accompanying their loss of turgor, must result in the passage of water into them from the mesophyll cells, thus again causing a rise in the suction pressure of the latter.

The first process implies a volume change in the tubes when they are



The effect of latex outflow upon the water uptake of a leaf of *Ficus elastica*. The rate of uptake was measured from 4.00 to 5.45 p.m. and then from 2.00 p.m. on the following day. Bleeding of latex was induced, at the times indicated by the arrows, by making cuts at the leaf-tip.

opened, the initial outflow of latex being accompanied by a relaxation of the distended tube walls, so that the mesophyll cells can expand by a similar amount. This is quite possibly the principal cause of the rise in the mesophyll suction pressure, and the time taken to reach the maximum—generally about one minute—then represents the time required for the outflow of sufficient latex to allow complete relaxation of turgor in the tubes.

The second process, which is discussed at length by Arisz and Frey-Wyssling, must also play some part, and would function equally well if the tubes were completely inextensible. Before the leaf-tip is cut the suction pressure of the latex tubes equals that of the surrounding cells, but as soon as the tubes are opened their turgor pressure falls and their suction pressure therefore rises by a corresponding amount, reaching a maximum value equal to the full osmotic pressure of the latex. This results in the passage of water into the tubes from the surrounding cells, so that the suction pressure of the latter increases. The latex is simultaneously diluted, and flows out from the tubes until its decreasing osmotic pressure, resulting from the dilution, equals the suction pressure of the surrounding cells, or until the ends of the tubes become closed. In the latter case a re-establishment of turgor in the tubes will occur.

In the present experiments the leaves have a free water-supply at the petiole end, so that the mesophyll suction pressure falls from the maximum at a decreasing rate to its original value. The fact that the cycle is repeated at subsequent cuttings, even in the case of the leaf bleeding under ammonia, indicates that at least partial closure of the tubes must occur, allowing re-establishment of turgor within them. In the experiment presented in the figure this must have occurred within seven minutes after the second cutting.

The progressive decrease in the maximum uptake rate at each bleeding is no doubt due to the lowering, by dilution, of the osmotic pressure of the latex remaining in the tubes so that they regain their original suction pressure, equal to that of the surrounding cells, at a turgor pressure progressively lower each time. If the tubes are extensible their final equilibrium volume will then be less after each bleeding, and the volume of the surrounding mesophyll cells will be correspondingly greater.

Experiments directed towards the further elucidation of the questions raised in this paper are now in progress.

SUMMARY

Potometer experiments with leaves of *Ficus elastica* show that outflow of the latex causes an increase in the rate of water uptake. This indicates an increase in the suction pressure of the cells of the leaf. The increase is progressively smaller at subsequent bleedings. The nature of the phenomenon is briefly discussed.

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Studies in the Vernalisation of Cereals. IV

The Effect of Preliminary Soaking of the Grain on the Growth and Tropic Responses of the Excised Embryo of Winter Rye

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With three Figures in the Text

IN a previous publication in this series (Gregory and Purvis, 1938) the question was raised as to the part played by the various tissues of the grain in the process of vernalisation. The successful vernalisation of *excised* embryos suggested that the embryo alone is concerned and that the effect of low temperature is confined to this organ. Two considerations, however, arise in this connexion: first, it has been shown that in the absence of a carbohydrate supply the isolated embryo cannot be vernalised by low temperature (Gregory and de Ropp, 1938); second, previous to the excision of the embryo in the work of Gregory and Purvis the grain had already been soaked in a sterilizing solution for a period of five hours. Although the preliminary soaking period has been shown to be of no importance as far as vernalisation is concerned, yet the work of Schander (1934), with maize, indicated that during the first few hours of soaking important changes occur in the grain which modify the growth of the embryo.

It therefore appeared necessary to study more closely the conditions obtaining in the grain during the first few hours of germination. These studies have been concerned with the effect which soaking grain for different periods of time has upon the growth and tropic responses of the excised embryo, and are the subject-matter of the present paper. The effect of removal or rupture of the aleurone layer, the changes occurring in the enzymatic activity of the embryo, and the changes taking place in the endosperm, have also received attention and results will be reported in later papers.

METHOD

The experiments here to be described utilize the method of growing isolated embryos first employed by Brown and Morris (1890) and later used by Gregory and Purvis (1936, 1938) in their studies on vernalisation.

In the experiments here described, winter rye (var. Petkus) was used
[Annals of Botany, N.S. Vol. III, No. 9, January 1939.]

exclusively. The removal of the embryos from the grain in a sterile condition was ensured by the use of aseptic rather than antiseptic methods. By first dipping the grain for a few minutes in 90 per cent. alcohol and then carefully removing, with sterile forceps, the integuments covering the embryo, it proved possible to cut out the embryo from the endosperm in an uninjured and sterile state; for the latter operation a flamed, spear-headed needle was employed. This method, if skillfully used, results in not more than 5 per cent. of contaminated embryo and obviates damage through the action of sterilizing agents.

The isolated embryos were grown separately on nutrient agar. Test-tubes $\frac{1}{2} \times 5$ in. were employed, each containing 7 c.c. of agar, the surface of which was steeply slanted. For satisfactory growth it was found sufficient to place the embryos with the scutellum resting on the surface of the agar, as the contact thus obtained was adequate. The medium was prepared by the addition of 3 per cent. sucrose to 0.7 per cent. agar, and mineral nutrients were supplied in the proportions recommended by White (1934) for the culture of excised root-tips. Sterilization of the solution was effected by autoclaving for ten minutes at fifteen pounds pressure.

The embryos were grown in a greenhouse at a temperature of about 20° C.

EXPERIMENTAL

1. Effect of previously soaking the grain on the growth of excised embryos in sucrose agar.

Table I gives the lengths of root and coleoptile attained after six days growth by embryos excised from grain which had been soaked for different periods.

TABLE I

Growth in Six Days made by Embryos excised from Grain after Varying Times of Soaking

Time of soaking (hr.).	Coleoptile length (mm.).	Root length (mm.).
0	32±3	57±12
2	58±3	97±10
8	60±4	100±6
24	21±5	44±12
48	11±3	20±8

Similarly in Table II are shown the effects of a fairly long period of preliminary soaking of the grain on the growth of the embryo in twenty days. In this experiment some of the tubes were kept in continuous darkness and others given alternating periods of twelve hours light and twelve dark. The embryos were grown on the usual 3 per cent. sucrose agar with mineral salts.

TABLE II

Effect of Fifteen Hours Soaking of Grain on Growth made by Excised Embryos in Twenty Days

LIGHT.	Root length (mm.).	Root No.	Coleoptile length (mm.).	1st leaf (mm.).	2nd leaf (mm.).
Embryos from soaked grain	206±16	3	21±3	83±5	104±11
Embryos from dry grain	110±18	3	14±1	73±5	75±13
IN DARK.					
Embryos from soaked grain	172±11	4	42±3	140±10	216±8
Embryos from dry grain	110±13	3	31±0	95±10	163±13

The most obvious fact which emerges from these tables is that the embryo may be removed directly from the grain before the latter is soaked and yet be capable of quite adequate growth. Embryos removed in this way grow into normal plants and reach maturity, producing ears and seed, thus indicating that the embryo, given a suitable supply of carbohydrate, is a complete biological system in itself and has no need of additional growth hormones from the endosperm.

It may also be seen, however, that the duration of the period in which the grain is soaked, prior to the embryo's removal to nutrient agar, has an important bearing on the subsequent growth. This soaking affects the growth both of roots and coleoptiles, but the relative effects remain almost constant. One interesting fact which emerges is the comparatively great rapidity with which soaking the grain produces its effect on the embryo. The factor or factors responsible for this acceleration of growth must pass into the embryo almost entirely during the first two hours of soaking the grain.

It appears from Table I that longer periods of soaking have a deleterious effect on embryo growth, though this result was not obtained consistently in these experiments and it is difficult to interpret. The deleterious effect of prolonged soaking was commented on by Schander (1934) who found that it could be prevented by removal of the external seed coats. According to Pringsheim (1930) it is to be attributed to the effects of intramolecular respiration, but recently Mosheov (1938) has extracted from wheat grains a germination-inhibiting substance.

Table II indicates that the factor from the endosperm affects embryos grown in the dark as well as those grown in the light, and continues to manifest its action even after twenty days when the second leaf is actively extending. It shows its presence also in other ways, for instance, the embryos excised from dry grain produce hardly any lateral roots, whereas in the embryos excised from soaked grain production of lateral roots is abundant. With embryos grown in the dark the leaves produced are more fleshy in the case of those excised from soaked grain, and are frequently tinged pink with anthocyanin which is completely absent from the embryos excised from dry grain.

As already stated the effect of preliminary soaking on the growth of excised

embryos was observed by Schander (1934) who worked, in this case, chiefly with isolated maize embryos. Since he added no sugar to the agar on which the embryos were grown his experiments were rather curtailed by the early starvation of the embryos.

2. Effect of soaking the grain on the response of excised embryos to gravity and light.

Since auxin is generally accepted as the proximal cause of both the geotropic and phototropic reactions, it is possible to test for its presence in

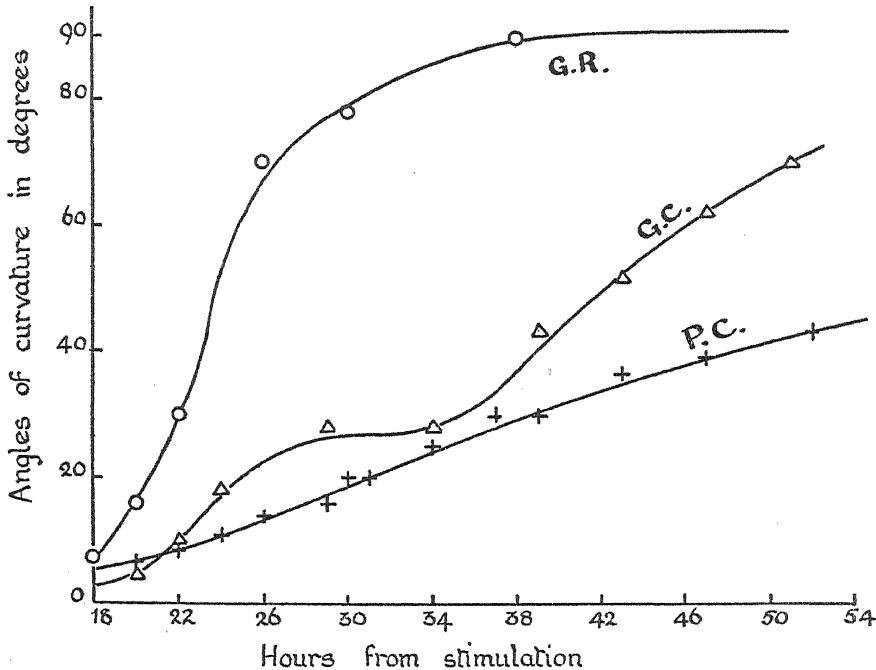


FIG. 1. The rate of curvature of coleoptiles and roots stimulated continuously from the time of excision of the embryos from dry grain. Embryos grown on agar with 3 per cent. sucrose. G = geotropic stimulation; P = phototropic stimulation; R = roots; C = coleoptile.

embryos by measuring the rate of their response to the stimuli of light and gravity. It may be stated at the outset that isolated embryos from dry grain have the power, during growth, of responding to both these stimuli. Furthermore, such embryos have this power even without an external sugar supply. Embryos excised from dry grain and placed on agar containing only mineral salts will show very definite coleoptile curvature after two-days growth if unilaterally stimulated from the outset. It may be mentioned here that the coleoptiles of embryos placed on nutrient medium without sugar always show a certain amount of extension growth, which, however, ceases after the first few days, from lack, presumably, of available carbohydrate. Preliminary

experiments indicate that, when this growth ceases, the capacity of the embryo to respond to stimuli also disappears.

It is only when embryos are grown on sucrose agar that the effect of previous soaking becomes clearly apparent, indicating the dependence of the tropic

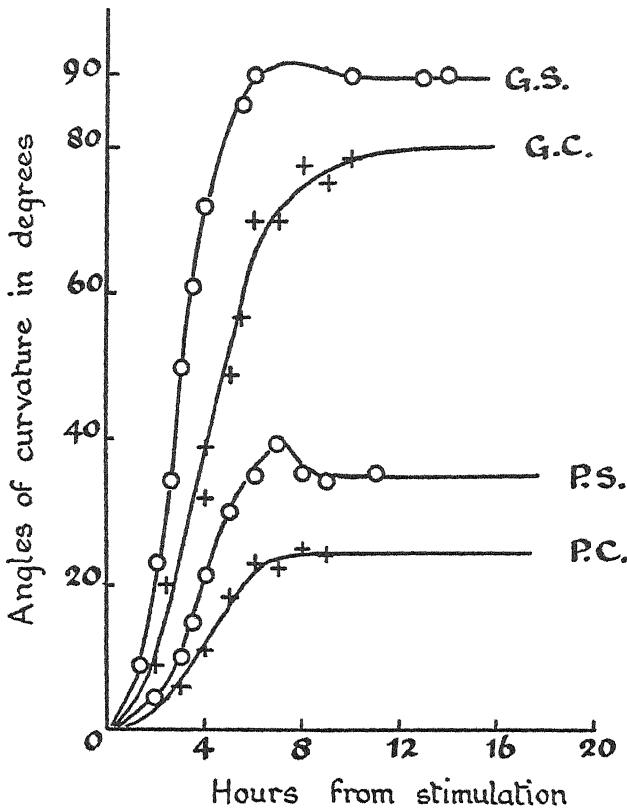


FIG. 2. The rate of curvature of coleoptiles of embryos excised from dry grain and from grain soaked for seven hours before excision, under stimulation beginning 3 days from excision. Embryos grown on agar with 3 per cent. sucrose and kept in the dark before stimulation. G = geotropic stimulation; P = phototropic stimulation; s = embryos from soaked grain; c = embryos from dry grain.

response on sugar as well as auxin, as has been demonstrated by several workers (Went, 1928, 1935; Schneider, 1938). Embryos placed on plain or sucrose agar and subjected at once to unilateral stimulation by gravity or light first begin to display curvature eighteen hours later. In this case the rate of response to both stimuli is relatively slow, the coleoptile having by this time attained a length of only 9 mm. and the roots of 7 mm. As may be seen from Fig. 1, the quickest response is that of roots to gravity, and the slowest that of coleoptiles to light.

If the embryos are allowed to grow in the dark for three days, before stimulation is applied, their rate of response is very different. By this time the coleoptiles have attained an average length of 40 mm. and their rates of curvature under phototropic and geotropic stimuli respectively are shown in Fig. 2. All the values in the above Figs. 1-2 are averages from ten replicates. Measurements of the curvatures were made by means of a horizontal microscope fitted with a cross wire in the eyepiece to which was attached a glass pointer moving over a circular scale. The embryos were grown in large test tubes on agar slopes. Light stimulation was applied by means of a 100-watt bulb at a distance of 1 foot.

As compared with the experiment in which the embryos were stimulated from the earliest stages of their growth (Fig. 1) the rate of response shown in Fig. 2 is greatly accelerated. Whether this is due to the presence of more available auxin, or to the greater length of the coleoptile, it is difficult to say. In Fig. 2 the effect produced by previous soaking on the response rate of the embryo is clearly evident. Embryos from grain soaked for seven hours curve both more and more rapidly than embryos excised from dry grain. The soaking does not seem to have affected the time for complete response and hence the final angle is greater in embryos after soaking.

A point of interest which emerges from these experiments is that the region in which response takes place is very narrow in the case of the rye coleoptile and moves upwards as the coleoptile increases in length. Thus a coleoptile in which curvature has been produced by unilateral exposure to light will, when the stimulus is removed, straighten out again under the influence of gravitation. It will not, however, straighten out at the same point at which it curved, but at a point higher up, and in this way exhibits a double curvature, the distance between the two bends being directly proportional to the time which has elapsed between the initial light and the subsequent gravitational stimulus.

It would appear from these experiments that even after three days in the dark, auxin has not disappeared from the coleoptiles or roots despite the fact that isolated embryos were used and very considerable growth had occurred.

3. *Combined effect of preliminary soaking and carbohydrate supply on growth of excised embryos.*

Among the factors controlling the growth-rate of excised embryos, two of the most important are the concentration of carbohydrate in the nutrient medium and the concentration of growth hormone in the embryo itself. In these experiments it is possible to control the supply of sucrose, while the concentration of growth hormone may be approximately controlled by varying the time of soaking the grain before the embryo is excised. In order to ascertain the extent to which these two factors affect the growth of the embryo, embryos removed from dry grain and from grain after two hours soaking were grown on agar containing 0, 0.1, 0.2, and 0.7 per cent. sucrose respectively.

The growth made by such embryos is shown in Fig. 3 from which it may be seen that even at the lowest concentration of sucrose (0.1 per cent.) a difference of hormone concentration, produced by duration of soaking, exerts a pronounced effect on the growth-rate of the embryo, both as regards coleoptile and roots.

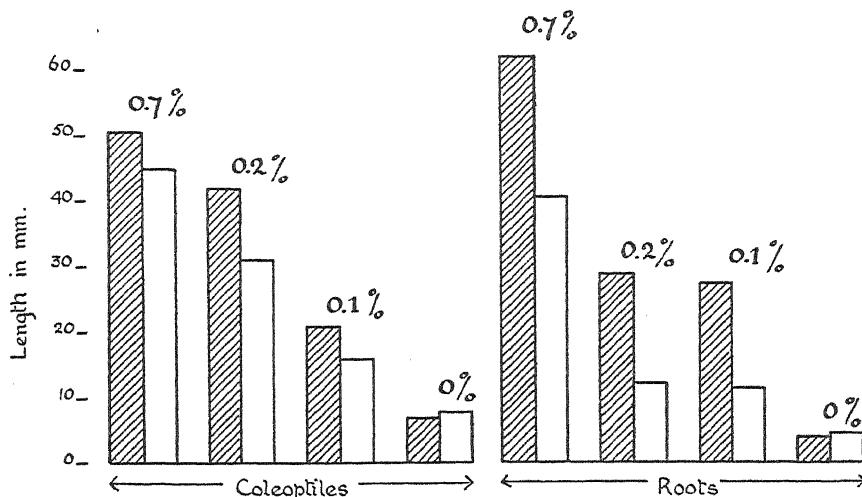


FIG. 3. Length attained in seven days of coleoptiles and roots of embryos excised from dry grain and grain soaked for 2 hours in the presence of various concentrations of sucrose in the nutrient medium (agar). Shaded columns refer to embryos from soaked grain and unshaded from dry grains.

These results are in qualitative agreement with the findings of Schneider (1938) who showed that the elongation of isolated coleoptiles is proportional to the sum of the logarithms of the sugar and hormone concentrations, i.e. to the product of the factors. The number of replicates used in this experiment (five) was insufficient to establish any precise relationship underlying this interaction effect. Nevertheless, it may be concluded from these experiments that neither hormone nor carbohydrate concentration can constitute a simple controlling factor in embryo growth. From Fig. 3 it would appear not unlikely that the effect of soaking is more pronounced on root than on coleoptile growth.

DISCUSSION

Several facts emerge clearly from the above experiments, the first of which is that the rye embryo, even when excised from dry grain, is a complete biological system capable of making normal growth without the acquisition of any growth substances from the endosperm. In view of this fact it becomes impossible to accept statements made by Pohl (1935-6) and Cholodny (1935) to the effect that the embryo is, by itself, devoid of the hormones necessary for growth and must receive these from the endosperm.

Secondly, it appears that some substance or substances from the endosperm enter the embryo during the very early stages of germination and exert a far-reaching effect on its subsequent growth. A significant aspect of these endosperm factors is that they affect root-growth to an even greater extent than that of the coleoptile, as regards simple elongation, and they affect also the production of lateral roots (section I). They also influence such processes as the production of anthocyanins in etiolated embryos.

In view of this multiplicity of effect it is difficult to suppose that the endosperm contributes only one growth factor to the embryo. Indeed, both from the work of Schander (1934) and from experimental results in this laboratory, not yet published, it may be deduced that the endosperm and aleurone layer constitute a complex interacting system which, in the intact grain, supplies the embryo with just that balance of growth factors necessary for optimum growth.

In this connexion the question arises as to whether it is possible to maintain that both root and shoot are controlled in their growth by one and the same hormone, i.e. 'auxin', or whether root-growth is controlled by some other factors. Thimann (1937, 1938) has suggested that the difference in the growth behaviour of shoot and roots depends only on quantitative relations to auxin. Thus he states: 'We are faced, therefore, with the fact that this one group of hormones, the auxins, brings about a variety of processes of growth and inhibition, and the effect they exert evidently depends on the kind of cell they enter and its physiological state.' Presumably the physiological state referred to would be greatly influenced by hormones of other types known to affect the growth of plant organs (Bonner, 1937).

Went (1938, 1938a) has considered in great detail the implications of the view that auxin is the ultimate controlling factor in plant growth. He, however, postulates the presence of specific growth promoting substances whose action is confined to certain organs of the plant, and whose distribution and translocation is ultimately controlled by auxin itself. The difference between these views turns, therefore, on a distinction between quantitative (Thimann) and qualitative (Went) relations.

In the case of the rye embryo, if it does in fact receive auxin from the endosperm, and if this auxin is the factor responsible for increased root-growth, it becomes evident on Thimann's view that the distribution of auxin in the embryo must be very minutely regulated. Root and shoot in the rye embryo are separated only by a small group of meristematic cells and the whole structure is, at the outset, only 2 mm. in length. But if Thimann's theory is correct the auxin concentration at the root end of the embryo is of the order of one-millionth of that of the coleoptile end.

It is difficult to see how such an unequal distribution could take place unless there is present in the isolated embryo another factor which both controls the rate of production of auxin and regulates its distribution. A 'regulation' of this kind would have something in common with the interaction

of the various 'calines' and auxin as postulated by Went. According to this theory the optimal growth of an embryo would take place only when the balance between 'regulator' and auxin is perfectly adjusted. It has been shown by Schander (1934), and confirmed by unpublished experiments in this laboratory, that a substance, not auxin, is supplied to the embryo by the aleurone layer and greatly influences the growth of the embryo. Possibly it is here that the source of the 'regulator' must be sought for.

On the view given above, what takes place when the grain is first soaked is a transfer both of auxin and 'regulator' to the embryo. The presence of this additional auxin would explain the increased rate of tropic responses noted in section II. The prolonged effect which preliminary soaking of the grain exerts on embryo growth cannot so readily be explained. There appears some evidence that, in these isolated embryos, auxin formation can take place by synthesis from sugar, and it seems that this synthetic activity can continue for a considerable period. In this connexion the experiments of Thimann and Lane (1938) on the effect of soaking the grain of oat in solutions of hetero-auxin are of some interest. Here also far reaching and long continued after-effects are noted, but in view of the fact that the outstanding morphological consequence of this treatment noted was a reduction in the length of the first leaf, whereas in this work the opposite effect holds (section I, Table II), it may be concluded that changes in auxin production are not the sole consequences of soaking the grain. On other points also this view has been reached.

In conclusion the author wishes to thank Professor F. G. Gregory, from whose assistance he has profited greatly, particularly as regards the theoretical considerations at the end of this paper.

SUMMARY

Rye grains were soaked for various periods and the effect on the subsequent growth of the *excised* embryo (grown on sucrose agar) studied.

Soaking the grain for as short a period as two hours produces increased growth of both roots and shoots in the excised embryo. This increase takes place irrespective of whether the embryo is grown in light or darkness and is still manifested twenty days after the excision of the embryo.

Production of lateral roots, both in light and darkness, and of anthocyanins in etiolated embryos is also increased by preliminary soaking.

Preliminary soaking of the grain has a marked accelerating effect on the tropic responses of the excised embryo.

Concentration of sugar and duration of preliminary soaking both effect the growth of the excised embryo, but neither constitutes a simple controlling factor in this growth.

It is concluded that during the preliminary soaking, both auxin and a 'regulator' controlling its production and distribution, enter the embryo from the endosperm and aleurone layer.

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NOTE

A SIMPLE MICROMANIPULATOR.—This instrument is designed to be used with a microscope for delicate dissections or manipulations of plant tissues. Among the existing types of instrument there seems not to be one which would lend itself readily to construction by the scientific worker himself with only moderate

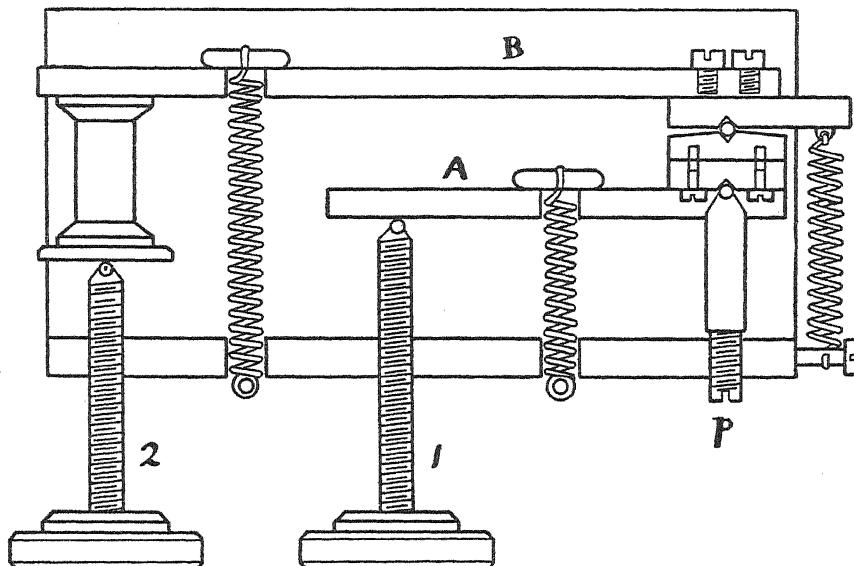


FIG. 1. Plan of micromanipulator. 2/3 full size.

skill and tool equipment. This instrument is designed to be so made. The necessary steadiness and precision are obtained by a massive baseplate in which all controlling screws are mounted, and by using bearings of kinematic design. Radial component movements at right angles in a horizontal plane are provided by a combination of two rocking levers. The vertical movement is linear and is obtained by sliding one of the levers up or down relative to the baseplate.

Construction. The general design is shown in the plan and elevation (Figs. 1 and 2). The baseplate is a piece of steel angle, 3 in. by 3 in. by $\frac{3}{8}$ in. by 7 in. long. Movement of the needle or pipette is controlled by two rigid T-shaped levers *A* and *B*, the needle being attached to lever *B*. The fulcrums upon which these levers rock are formed by steel balls $\frac{1}{8}$ in. diameter which engage in grooves filed on the vertical members. The grooves may easily be cut by hand by first making a slot with a slitting hacksaw blade and then by filing with a 60° angle triangular file deep enough for the balls to seat well upon the bevel, as shown in the enlarged drawing (Fig. 2). Upon the accurate cutting of these grooves depends very largely the satisfactory working of the instrument. In the experimental instrument

constructed by the author the grooved members are of brass, but it would be better to use carbon steel so that they could be hardened to reduce wear. Lever *A* has a vertical groove which engages the ball-ended pegs (*p*) in the baseplate, and another

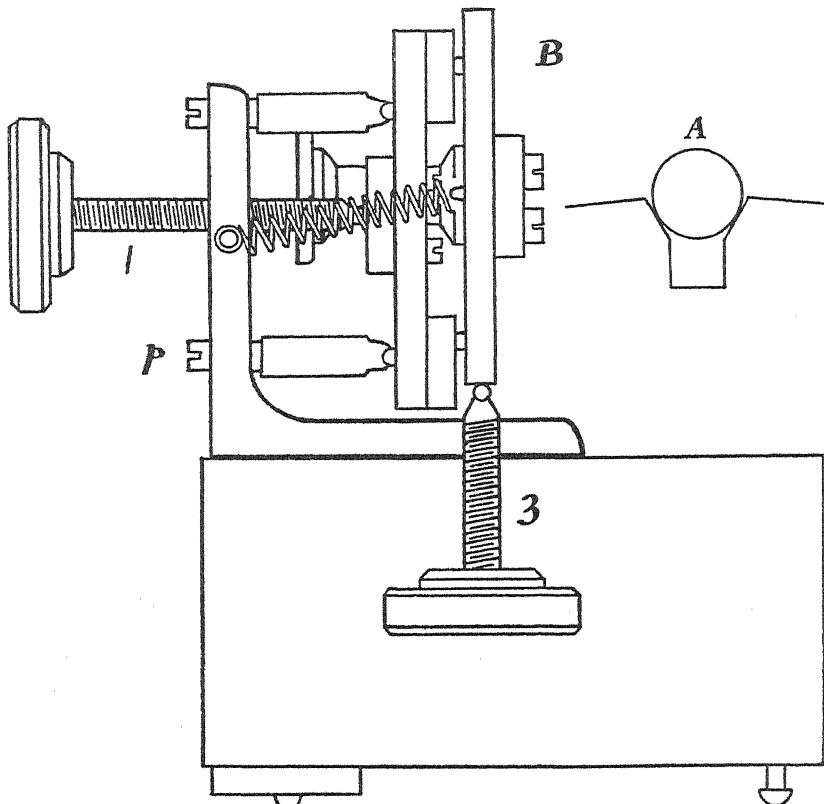


FIG. 2. Side elevation. 2/3 full size. A. Enlarged diagram of ball-bearing.

groove opposite to it which forms, together with a similar groove on lever *B*, a race for two balls which allow lever *B* to rock about a vertical axis upon lever *A* and also to move vertically relative to it. Levers *A* and *B* are controlled by the screws 1 and 2 respectively ($\frac{1}{4}$ in. diam. and 40 threads to the inch), which work in the front of the baseplate. By rotating screw 2 lever *B* is caused to rock about the ball-race between it and lever *A*, so that the needle moves across the microscope field towards or away from the operator. If screw 1 is turned lever *A* rocks upon its fixed fulcrum and displaces that of lever *B*, so moving lever *B* forwards or backwards and causing the needle to move laterally in the field. By operating the screws together any combination of these movements in a horizontal plane can be imparted to the needle. Vertical movement is provided by the screw 3, upon the point of which the vertical member of lever *B* rests. Backlash is avoided by having the parts of the apparatus held together by fairly strong springs in tension, which are arranged as shown in the drawings.

It is essential for the successful working of a micromanipulator that the mountings, both of the instrument itself and of the microscope, be as rigid as possible. The method of mounting the experimental micromanipulator may be seen from the figures. The baseplate is screwed to a wooden block which is mounted on a metal frame having three studs to give a tripod support. In place of the studs levelling screws could be fitted which would allow adjustment to microscopes of different stage height. It has been found that lack of rigidity in the laboratory bench itself is a frequent source of trouble in using the micromanipulator, even a teak bench, 2 in. thick, as usually supported, being insufficiently rigid for delicate work. A slab of slate or marble, suitably mounted on a strong bench, usually forms a sufficiently rigid platform.

The needle may be mounted upon the lever *B* with 'Sira' wax, and metal clamps may be bolted to the lever to hold micropipettes or other special instruments. Glass needles can with a little practice be made from rod by first drawing it out to about half-millimetre diameter and then, by heating the middle locally with a hot platinum wire and drawing gently, two finely pointed needles can be obtained. This gives a strong-shanked needle which tapers abruptly to a sharp point.

The author's experimental instrument has been successfully employed by Heath (New Phytol., in the press) in an investigation of the mechanism of stomatal movement, and by Spencer (1939, Ann. Bot., N.S., iii. 227), who studied the effect of puncturing individual latex tubes of *Euphorbia Wulfenii*.

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Chromosome and Chromonema Length during Meiotic Coiling in *Trillium erectum* L.

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With Plates V and VI and one Figure in the Text

INTRODUCTION

SO far as we are aware, the only attempt yet made to gain any detailed knowledge of chromosome lengths throughout meiosis was that by Belling (1928), who showed that the chromosomes in *Lilium* (*L. Pardalinum*, *L. longiflorum*, and *L. regale*), as well as in *Aloe purpurascens*, lengthen from leptotene to pachytene and then contract until diakinesis, when they are only about one-tenth as long as at pachytene. It was further shown that there was little or no change in chromosome length from late diakinesis to anaphase. It must be emphasized that Belling was dealing with chromosome length, not chromonema length. Probably, if allowance is made for bends and chiasmata, these two are synonymous up to the time when the chromonema begins to assume a large-gyred spiral structure (the 'major spiral' of Huskins and Smith, 1935, Darlington, 1935, and others). After this the 'chromosome length' becomes the distance from end to end of the coil (or of the 'bulk-stained' chromosome) without reference to the distance around the gyres of the spiral, which is the 'chromonema length'.

In the present study of *Trillium erectum* L. undergoing meiosis at various temperatures the length of the chromonema was determined at stages from early diakinesis, when major coiling begins, to the end of the heterotypic anaphase, when the coil has reached its maximum compactness. One set of measurements was also made at the second anaphase.

MATERIAL AND METHODS

The data were gathered from the following sources:

1. Huskins and Smith's (1935) publication on the structure of normally synaptic chromosomes of *Trillium*. These underwent meiosis in a greenhouse at 18°–20° C. This will be referred to as 'standard' material.

2. Hunter's (unpublished) studies on normal and asynaptic Trillium. These are cited only in the discussion.
3. Original observations on desynaptic chromosomes occurring in material kept from early meiotic prophase in a constant temperature chamber at 9°–10° C. in 1936.
4. Normal and abnormal non-coiled chromonemata in material kept in a constant temperature chamber at 18°–19° C. in 1936.
5. Normally paired and coiled chromosomes in material kept from pre-leptotene in a constant temperature chamber at 12°–13° C. in 1937.
6. Normally paired and coiled chromosomes in material kept in a constant temperature chamber at 16°–17° C. in 1937.
7. Abnormal non-coiled chromonemata in material kept in a constant temperature chamber at 20° C. in 1937.

General physiological factors, were undoubtedly involved in producing the various abnormalities, as well as temperature *per se*. In 1937 greater care was taken to avoid fluctuations in temperature between the time of collecting the corms in the open and placing them in the temperature chambers. They were also collected earlier in 1937 than in 1936, all being pre-leptotene in 1937. The 1936 material was partly at leptotene and partly pachytene and early diplotene when collected. These factors probably account for the greater irregularity in the 1936 material.

Observations were made with 2 mm., 1·4 N.A. and 1·5 mm., 1·3 N.A. objectives. Measurements of chromosome length were made in part directly with a 10× micrometer eyepiece combined with either one of these objectives. Chromonema lengths were all measured from camera lucida drawings.

OBSERVATIONS

Material (1): Measurements of chromosome lengths near the beginning of, and after completion of, spiralization in standard Trillium were made from Huskins and Smith's (1935) Plates I and II, figs. 8 *a*, *b*, *c*, *d*, 9, 10, 12, 16, 17, 18, and 19. These were then checked by direct measurements on the original preparations. Since the agreement between these two sets of values was very close, as shown in Table I, we feel justified in assuming that the various calculations made from the published figures are reliable. The measurements show that there is little alteration in chromosome length during spiralization. In neither of these sets of measurements was account taken of minor bends in the general outline (pellicle) of the chromosome, nor of contortions due to chiasmata; they are crude chromosome lengths such as would be obtained from ordinary 'bulk-stained' preparations in which internal structure is not revealed.

Measurements of chromonema lengths made from the same figures are presented in Table II. While it is relatively easy to take into account minor contortions in measuring a chromonema which is not coiled, determination of the length of a coiled chromonema presents serious difficulties. Measure-

ment of the coil in one plane (as though it were a zigzag line) is subject to the error involved in ignoring the depth of the gyres and obviously gives an underestimate. The alternative to this method, applying the mathematical formula for the length of a spiral, while theoretically correct, is in practice subject to the limitation that slight errors in measuring the diameter of the gyres leads to appreciable errors in the calculations of the spiral length. Both methods have been used throughout this work. It must be emphasized that all the lengths cited involve considerable possibility of error, on account of natural deviations in different chromosomes and different cells, of possible variations in fixation, and of inevitable errors in measurement, particularly in small samples. Conclusions in this and other papers will therefore be drawn only from differences that are of considerable magnitude. It will be seen from Table II that there is a very distinct increase in the length of the chromonema during the process of coiling. Tables I and II summarize the results of

TABLE I

A Comparison of Chromosome Lengths near the Beginning of, and after Completion of, Coiling. Standard Material (1)

Chromosome.	Chromosome lengths (μ).			
	At beginning of coiling.		After coiling.	
	Measured from slides.	Measured from drawings.	Measured from slides.	Measured from drawings.
B	12.6	12.7	13.4	12.5
C	9.1	10.0	10.6	10.0
D	12.4	13.6	12.7	13.0
E	15.5	15.5	16.5	16.4

TABLE II

A Comparison of Chromonema Lengths after Beginning of, and after Completion of, Coiling. Standard Material (1)

Chromosome.	Near beginning	Partially	Coiled.	Calculated	Mean.
	of coiling.	coiled.			
	Measured from drawings.	Measured from drawings.	Measured in one plane.	from formula for spiral.	
B	17.6	20.3	28.7	49.7	39.2
C	12.9	18.7	20.7	38.1	29.4
D	23.5	—	24.3	40.8	32.5
E	30.4	—	45.4	73.8	59.6
Total	84.4	—	119.1	202.4	150.7

Number of gyres (per 5 chromatids) = 58.0.

Diameter of anaphase gyres = 1.5μ

Ratio of anaphase to diakinesis chromonema lengths:

(a) Anaphase lengths measured in one plane	1.41
(b) Anaphase lengths calculated from formula	2.39
(c) Mean of (a) and (b)	1.90

measurements and calculations on the four chromosomes designated B, C, D, and E by Huskins and Smith (1935). (Their figures do not include an uncoiled A chromosome.) Also included in Table II are the chromonema lengths of one B and one C chromosome which were only partially coiled. It will be noted that they are intermediate between those of the slightly and fully coiled.

Material (2) and (3): Hunter's data on chromosome length and changes in the direction of coiling of the chromonema as well as our earlier observations on desynaptic material are cited in the preceding paper of this series (Huskins and Wilson, 1938). Their significance will be considered further in the discussion (p. 817).

Material (4): In this material a number of bivalents were found which had apparently reached late diakinesis without the formation of major coils. The homologues were widely separated, with well-defined chiasmata between them. In some cases these appeared to be in process of terminalization. Both the age of the material and the lengths of the chromosomes rule out the possibility of their being early stages. Measurements on twenty-three normal and seven abnormal chromosomes occurring on the same slide showed no significant difference in chromosome length (Table III). The chromonema

TABLE III
Comparative Lengths of Chromosomes having Non-coiled and Coiled Chromonemata. Material (4)

Chromosome.	Length of non-coiled (μ).	Length of coiled (μ).
A	6.8	7.5
B	8.7	8.7
C	6.2	8.1
D	—	8.7
E	12.0	15.0

lengths of the coiled material must, of course, have been very much greater than those of the non-coiled, which have evidently not elongated.

Material (5): The data from this material kept at 12°–13° C. are presented in Table IV. In it the chromosome length was similar to that of the standard (Table I). The chromonemata are shorter. The mean number of gyres in the standard material is 58 (Table I, Huskins and Wilson, 1938), and in the present material it is 43. The mean inter-gyre length is therefore greater in the latter, which accounts for what may at first sight appear to be a discrepancy in the ratio between the one-plane and spiral measurements of the two. Minor, but real, discrepancies may have arisen from the fact that independent measurements were made in arriving at the one-plane and spiral figures. Further, in the standard material diakinesis chromonema lengths were determined when coiling was just beginning, while in material (5) it was slightly further advanced.

Material (6): The data from this material kept at 16°–17° C. are presented in Table V. The chromosome lengths are greater than in either the standard

TABLE IV

Comparison of Chromosome and Chromonema Lengths at Diakinesis and First Anaphase. Material (5)

Chromosome.	Chromosome length (μ).		Chromonema length (μ).		Mean.
	Diakinesis.	Anaphase.	Diakinesis.	Anaphase.	
A	10.0	10.0	12.7	16.2	25.6
B	11.0	10.0	13.7	20.8	27.8
C	8.2	8.5	10.0	14.7	22.4
D	10.2	12.5	13.5	20.5	32.0
E	14.0	16.0	20.0	27.2	46.5
Total	53.4	57.0	69.9	99.4	154.3

Number of gyres (per 5 chromatids) = 43.0.

Diameter of anaphase gyres = 1.5 μ .

Ratio of anaphase to diakinesis chromonema lengths:

(a) Anaphase length measured in one plane	1.30
(b) Anaphase length calculated from formula	2.88
(c) Mean of (a) and (b)	2.09

TABLE V

Comparison of Chromosome and Chromonema Lengths of Mid-Diakinesis and First Anaphase in Material (6)

Chromosome.	Chromosome length (μ).		Chromonema length (μ).		Mean.
	Diakinesis.	Anaphase.	Diakinesis.	Anaphase.	
A	12.0	11.4	15.4	20.3	28.0
B	13.7	13.2	16.4	18.9	31.9
C	12.5	10.9	15.7	17.4	27.5
D	14.2	13.7	16.7	20.4	30.6
E	21.4	17.1	24.3	34.8	47.6
Total	73.8	66.3	88.5	111.8	165.6

Number of gyres (per 5 chromatids) = 42.0.

Diameter of anaphase gyres = 1.5 μ .

Ratio of anaphase to diakinesis chromonema lengths:

(a) Anaphase length measured in one plane	1.26
(b) Anaphase length calculated from formula	2.48
(c) Mean of (a) and (b)	1.87

or the 12°-13° C. material. The chromonema lengths are slightly less than those of the former, and greater than those of the latter. The mean number of gyres is 42, compared with 58 and 43. The diakinesis chromosome and chromonema lengths were, perforce, measured at a slightly later stage than

in the other material. The difficulty of getting strictly comparable stages in all the experimental materials will of course be realized. This applies particularly to prophase stages and is least serious at anaphase.

Material (7): This material was kept at 20° C. The data are presented in Table VI. Elongation of the chromonemata has occurred, but they have not

TABLE VI

Comparison of Diakinesis and First Anaphase Chromosome and Chromonema Lengths in Material (7)

Chromosome.	Chromosome lengths (μ).			Chromonema lengths (μ).		
	Early Diakinesis.	*Mid- Diakinesis.	†Ana- phase	Early Diakinesis.	Mid- Diakinesis.	Anaphase.
A	—	15·8	24·1	—	17·1	25·8
B	12·8	17·5	22·6	13·4	19·2 [‡]	24·2
C	—	15·0	16·4	—	16·7 [‡]	18·9
D	17·5	20·0	24·3	18·8	21·0	24·3
E	18·5	26·5	36·3	18·9	30·0	37·5
Total	—	94·8	123·7	—	104·0	130·7

Ratio of mid-diakinesis to first anaphase chromonema lengths = 1·3.

* All from one cell.

† All from one cell except the B chromosome.

‡ In these two chromosomes the chromonema lengths could not be determined accurately; the values were therefore calculated by adding to the chromosome lengths the mean difference between the chromosome lengths and the chromonema lengths of the other chromosomes in the cell.

coiled. Only a very few cells were sufficiently clear for detailed study. There were, however, some hundreds of cells on the best slide obtained, and of these the great majority had their chromonemata completely non-coiled, a few at diakinesis showed zigzags, and one single anaphase cell had more or less normal coils. The amount of elongation determined from comparison of anaphase chromosomes with those judged to be at about mid-diakinesis is less than in any of the other material, excepting, of course, those cells in material (4) which entirely lacked elongation. The few chromosomes which could be measured at early diakinesis were, however, considerably shorter; elongation is therefore probably about normal in this material.

THE DIRECTION OF COILING

The direction of coiling was analysed, gyre by gyre, in normal synaptic, desynaptic, and synaptic material in an effort to determine whether or not association affects the direction of coiling. In three A, five B, three C, four D, and four E bivalent chromosomes of standard synaptic material (materials 1 and 2) it was found that three gyres coiled in one direction and one in the other in 38 per cent. of the gyres analysed. In two cells of desynaptic material (material 3) 59 per cent. of the gyres showed three chromonemata coiling in one direction and one in the other, and, in an asynaptic cell (material 2) in which there was no association between either homologous chromosomes or

sister chromatids (asynaptic type 2), about 50 per cent. of the gyres showed this condition. Since, if coiling were completely random, 50 per cent. of the gyres would be of the three and one type, the above figures suggest that direction of coiling is random in this type of asynaptic chromosome, but not quite random in normal synaptic material and possibly not in desynaptic.

In addition to this Dr. A. W. S. Hunter (1937 and unpublished) has found that in asynaptic material in which there is close association between sister chromatids (asynaptic type 1), the direction of coiling is always the same in such associated strands. The direction of coiling in homologues seems to be random in this case.

It appears that in *Trillium erectum* the major coil exists very nearly unchanged from its inception at early diakinesis to the second telophase, and

TABLE VII

Length and Number of Gyres of Second Anaphase Chromosomes from Material (6)

Chromosome.	Chromosome length.	Number of gyres.
A	8·7	6
B	10·0	7
C	8·7	6·5
D	11·2	7
E	16·2	11·5
Total	54·8	38·0

Gyre diameter = 1·5 μ .

that uncoiling is completed during the first pollen-grain prophase. Second division chromosomes suitable for analysis are rare. In material (6), however, it was found possible to obtain one clear example each of chromosomes A, B, C, D, and E. These were analysed for chromosome lengths and number of gyres (Table VII), and it was found that both were slightly less than at first anaphase of the same material. The gyre diameter was the same.

DISCUSSION

Various theories of chromonema coiling have been advanced by a number of authors. None of them appear to be compatible with the data here presented. Four of the more recent ones will be considered, and also a new hypothesis suggested by the data. We would emphasize in advance, however, that in our opinion formulation of any reasonably complete hypothesis of coiling must await further knowledge of the physico-chemical nature of the chromosomes and of their relationships with the other contents of the cell. 'Matrix' and 'pellicle' or 'sheath' are still almost unknown quantities. The present data bring some evidence both direct and indirect to bear upon them.

Kuwada (1927) makes the suggestion that the spiral may be due to the tension set up by the further contraction of the chromonemata after the chromomeres have come into contact with each other. This would, of course, involve shortening during spiralization. Further, if such contraction should

take place it would be reasonable to suppose that the chromomeres might move out of their straight alinement into a zigzag arrangement, but it does not seem probable that a spiral of such a pitch as is observed in *Trillium* could arise in this way.

Darlington (1935) presented a frankly deductive theory of chromosome coiling based on the assumption that a molecular spiral gives rise to the visible spirals which must be in the opposite direction to the internal twist. If this were so, both the chromonemata and the chromosomes should shorten during spiralization. Belling found that the chromosomes remain practically stationary in length in *Lilium* and *Aloe* from late diakinesis to anaphase, which is probably the period during which spiralization is completed in these plants, and evidence presented in this paper shows that there is little change in length of the chromosomes of *Trillium* from the beginnings of major spiralization to its completion. Our data show that the chromonemata lengthen during spiralization. Apart from this evidence against the torsion theory is the fact that it is impossible to explain changes in direction of coiling satisfactorily on it, and such changes are very frequent in *Trillium* (cf. Huskins and Smith, 1935, and Huskins and Wilson, 1938).

Huskins and Smith (1935) suggest that growth in thickness occurs on the outside of the chromatid and, therefore, if the half-chromatids are twisted around each other, such growth must be occurring in a spiral. If syneresis follows it would result in a tension which would in turn favour the assumption of a zigzag or spiral form. They suggest also that the spiral growth may itself produce a spiral form. Though the present evidence shows that syneresis is not occurring during spiralization and this hypothesis cannot, therefore, be valid in its simplest formulation, the factors it invokes must be considered in the formulation of a more complete hypothesis.

The simplest hypothesis so far advanced is that of Sax and Humphrey (1934). They point out that two closely associated threads compressed within an enveloping pellicle in such a way that the ends are not allowed to rotate would form a spiral of the type required. As expressed, their idea necessarily supposes a contraction of the chromosome as a whole while the chromonema length remains constant, neither of which suppositions are true for *Trillium*. The factors invoked must, however, be considered, especially if *Tradescantia* is shown to differ in this regard.

The present data lead us to formulate the following as the most reasonable conclusions: (1) the chromonema is elongating during spiralization; (2) under conditions abnormal for *Trillium* (material 4) both elongation and coiling may fail to take place; (3) the number of gyres of a chromonema is a function of its length and of the chromosome diameter (see Huskins and Wilson, 1938); (4) the amount of randomness in the direction of coiling is dependent on the closeness of association of the chromatids during spiralization; (5) the major coil persists almost unchanged from its formation at diakinesis to its unravelling at first pollen-grain division prophase.

Leaving all other points aside for the moment, it seems probable that the correlation between elongation and spiralization indicates a causal relationship; any hypothesis on the mechanism of coiling must take this correlation into account. An hypothesis which emerges directly from these data is that spiralization results simply from the elongation of the chromonema within a limiting boundary.

As the simplest possibility, let us consider the space-limiting factor to be a pellicle¹ which remains nearly stationary in size while the chromonema is elongating. Let us further assume, in accord with morphological evidence (apart from functional considerations—cf. Berrill and Huskins, 1936), that this pellicle forms prior to the beginning of spiralization and in some manner disappears prior to the unravelling of the spiral in first pollen-grain division prophase.

Some of the expected properties of the spiral which this method of formation would produce are:

1. If elongation is gradual and uniform throughout the length of the chromonema and coiling is *not* caused by an internal torsion, an irregular zigzag would be expected in the early stages of spiralization.
2. Any point of interruption of the spiral such as chiasmata, the attachment region, bends in the pellicle, or any lack of homogeneity in the chromonema and its matrix, might cause changes in the direction of coiling (with random frequency).
3. Chromatids, closely associated during spiralization, should coil in the same direction.
4. Uncoiling would be expected to be gradual.

Do the observed properties of the major spiral in *Trillium* agree with these expected properties? Concerning the first, reference may be made to Huskins and Smith's (1935) paper in which mention is made of the irregularity of the coil at the beginning of spiralization. Plate II, figs. 8, 10, and 13 of that paper show stages from early zigzag to the relatively regular spiral. The zigzag of intermediate stages is also shown in the diakinesis chromosomes illustrated in this paper. It will be noted that the anaphase chromosome spirals are not perfectly regular. When we consider, however, that there has been a considerable amount of contortion due to chiasmata and other causes, such irregularities are expected. More regularity would be expected in asynaptic chromosomes which have no chiasmata to interfere with coiling. Dr. A. W. S. Hunter has observed this.

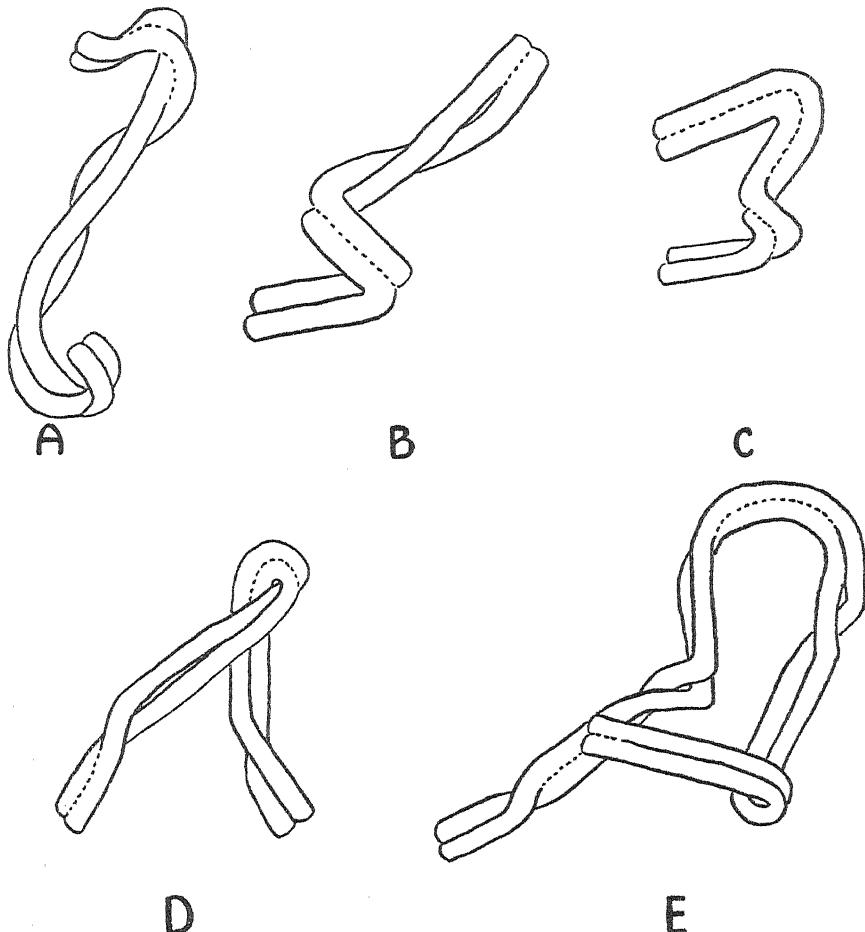
Concerning the second expected property, it was shown in our previous paper (1938) that changes in direction of the major coil were probably due to three factors: chiasmata, the attachments, and some factor associated with the number of gyres. That the first two factors would influence the direction

¹ Darlington, who, contrary to us, doubts the existence of a 'matrix', agrees (1935a) that: 'There are several grounds—chiefly non-morphological—for assuming that the chromosome thread has some sort of pellicle.'

of coiling on the suggested method of spiralization is quite obvious. The third factor is probably a more complex one. As already mentioned, any bend in the pellicle might affect the direction of coiling, and frequency of bends is probably proportional to length. Further, it is possible that coiling begins at various places along the chromonema. In any region where it may begin, the direction of coiling will, on our hypothesis, be random. The longer the chromosome, the greater the chance, therefore, of oppositely coiled segments. There remain also unknown structural factors which may affect the coiling process.

With regard to the third expected property of some correlation between the amount of randomness in the direction of coiling of the four chromatids of a bivalent and their closeness of association, it has already been pointed out that where there is no association (asynaptic type 2) randomness is complete; where there is some association (normal synaptic material with chiasmata) randomness is not quite complete; and where there is complete association of chromatids (asynaptic type 1) the direction of coiling of the associated threads is the same. The desynaptic material may seem to be an exception to this rule, but consideration of the process of desynapsis shows that it is not necessarily so. It is true that since there were chiasmata in this material there must have been at one time a certain amount of association of the component threads of the bivalent, and therefore it might be expected that randomness in the direction of coiling would not be complete. The chromosomes have, however, pulled apart, and the chiasmata been resolved much earlier than normal (apparently by passing off the ends). It is therefore probable that at the time of spiralization the only association of chromatids would be at their ends, and this would involve only a few gyres. If this were so an approach to randomness in the direction of coiling would be expected.

With regard to the rate of uncoiling of the chromonema, it may be said that it seems to be relatively slow since it is not complete at a fairly late stage of the pollen-grain division prophase (in the text-figure). This indicates a consistency compatible with coiling rather than with folding which might occur on elongation. A further point which must be considered is whether or not the unravelling of a longitudinally double spiral (a chromatid spiral having a 'tertiary' split), such as is observed by Huskins and Smith (1935) and others, would result in the configurations taken up by the chromatids in first pollen-grain division prophase. A certain amount of intertwining has been observed, and whether or not this could result from the unravelling of a coil after the 'tertiary' split is debatable. The answer, in part at least, would probably depend upon the plane of the tertiary split, of which we have no certain knowledge. It is probably significant that all the intertwining which has been observed in the material which was used in the preparation of this paper, as well as most of that shown by Huskins and Hunter (1935), is due only to the fact that uncoiling was incomplete; it could disappear when the threads were completely straightened out. The relation of the major coils of meiosis to



A-E. Pro-metaphase chromosomes at first pollen-grain division. Major spiral not completely uncoiled. Regions where doubleness is not clear are indicated by dotted lines. $\times 2,500$.

the 'relational' (Darlington, 1935) coils of mitosis has not yet been studied inductively-deductively, nor has any rigorous analysis of Darlington's deductive hypothesis been published. Further, though it is well established that chromatids are often intertwined in the prophase of mitosis (see especially Sax and Sax, 1935), satisfactory evidence has yet to be presented that 'relational coiling' occurs in the prophase of meiosis. Finally, the nature of the meiotic 'minor spiral' has not yet been established satisfactorily. We are still unable to see more than a waviness along the chromonema in *Trillium*, as reported earlier (1935), and are of the opinion that many illustrations of minor spirals in meiosis are the result of optical illusions or are misinterpretations of the major spiral under various conditions.

It seems clear from the foregoing discussion that uniform and gradual elongation of the chromonema within a confined space can readily account for the type of spiral observed in meiotic chromosomes of *Trillium*. The nature or the mechanics of this elongation must, however, remain an open question. So also must the nature of the pellicle. We can say as a result of direct observation that the cause of elongation must act with uniformity throughout the length of the chromonema, and also must act gradually since all evidence points to a gradual lengthening rather than to an abrupt change. It is clearly not terminal growth.¹ Future studies of chromonema spiralization and chromosome mechanics in general must, of course, attempt an explanation in terms of organic molecular structure and patterns, our present inadequate knowledge of which, however, should not be allowed to prejudice the analysis of readily observable structures. We are here emphasizing the readily observable features and leaving such controversial structures as minor spirals, which are at or below the limits of clear optical definition, for further study and separate discussion. It is possible that a deductive approach from indirect evidence will be essential. If so, we shall endeavour, as in the present paper, to distinguish sharply between direct and indirect evidence, and as far as possible to keep hypothesis in its place as a guide rather than as an interpretation.

SUMMARY AND CONCLUSIONS

From early diakinesis to late anaphase, during which time the major coil is formed in meiotic chromosomes of *Trillium erectum* L., the chromonema about doubles in length while the chromosome length remains almost

¹ Astbury (1934) has shown that protein fibres of hair keratin may be reversibly stretched from the normal state in which the main peptide chains are regularly folded in planes lying transverse to the side chains to a state in which the foldings disappear. This about doubles their length. Since the chromonema may likewise double its length during coiling, it is possible that the observed lengthening may be due to a similar molecular reorientation. The pellicle may be an interface at which a change of surface tension might account for the stretching. It is, however, well to bear in mind that reversible changes in length can take place in all fibrous proteins, notably those of feather, keratin, and muscle fibres (myosin) without any evidence of intra-molecular reorientation appearing in the X-ray diagrams. We are indebted to Dr. B. R. Nebel and Professor D. L. Thomson for suggesting and criticizing these and other possibilities.

Those who believe that the meiotic chromosomes have a rather tightly wound minor coil (not a mere waviness) running along the length of the major coil generally assume both chromonema and chromosome elongation and contraction to be due chiefly or solely to spiralization. They are, however, divided in their interpretation of the method of development of the major coil. Kuwada (1938), for instance, considers that in *Tradescantia* there is a minor spiral in early prophase which expands into the major spiral by diakinesis and that a new minor spiral develops along it. Darlington (1937) interprets the same and other observations as showing the large-gyred spiral being superimposed upon the small-gyred one. 'This secondary coiling accounts for the greater reduction in length of meiotic chromosomes.' Until this point is cleared up and measurements are available for *Tradescantia* or until it is clearly shown that there is, contrary to our present opinion, a minor spiral more or less closely coiled at some stage in *Trillium* meiotic chromosomes, it is scarcely profitable to discuss the possibility of a minor spiral being concerned in the demonstrated elongation of the *Trillium* chromonema during formation of the major spiral. There are differences between *Trillium* and *Tradescantia* which are now receiving detailed consideration.

unchanged. Though the individual measurements must be subject to a rather wide margin of error, their general consistency in materials developing under widely different experimental conditions renders this conclusion inevitable.

Torsion hypotheses of spiralization have (herein and previously) been shown to be incompatible with observation on changes in the direction of coiling. The present observation of elongation appears definitely to render invalid all previously advanced spiralization hypotheses.

While sufficient knowledge of the structure of the chromonema is not yet available for the formulation of any comprehensive hypothesis of spiralization, the primary observational data suggest that coiling is due to elongation of the chromonema within an enveloping sheath or pellicle, the nature of which also remains to be determined.

This simple hypothesis fulfils, however, various expectations on data gathered from normal synaptic, desynaptic, and two types of asynaptic material, and also from other abnormal material in which elongation took place in the absence of a pellicle and coiling did not occur.

While spiralization can no longer be looked upon as a general process of contraction or syneresis, it may still be viewed as a mechanism of chromosome contraction *relative to* chromonema length. Spiralization within a pellicle enables anaphase separation of the elongated chromonemata to take place without entanglement across the plate.

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DESCRIPTION OF PLATES V AND VI

Illustrating Mr. G. B. Wilson and Professor C. L. Huskins's paper on Chromosome and Chromonema Length during Meiotic Coiling in *Trillium erectum* L.

PLATE V

Magnification 4,000 reduced to 2,700 in reproduction excepting Figs. 3 and 4 which are $\times 3,500$ reduced to 2,300 in reproduction.

Figs. 1-5. Chromosome complement of a cell from material (5) at early diakinesis. As shown, coiling has already begun.

Figs. 6-10. Chromosome complement of a cell from material (5) at first anaphase.

Figs. 11-15. Chromosome complement from a cell of material (6) at mid-diakinesis. Note that the C-chromosome is almost completely coiled.

Figs. 16-20. Representative pairs of first anaphase chromosomes of material (6).

PLATE VI

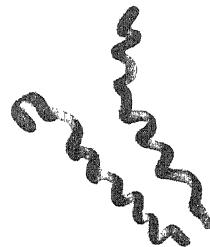
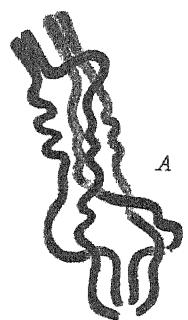
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Figs. 21-3. Early diakinesis chromosomes from material (7).

Figs. 24-7. Mid-diakinesis chromosomes from material (7).

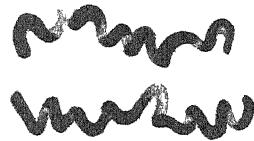
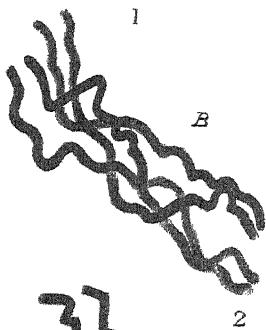
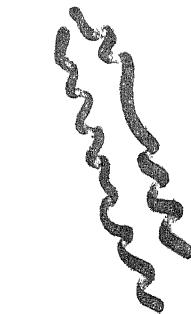
Figs. 28-32. Pairs of anaphase chromosomes from material (7). All except Fig. 29 are from one cell.

Figs. 33-7. Second anaphase chromosomes from material (6). Figs. 33, 34, and 35 are from one tetrad and Figs. 36 and 37 from another.

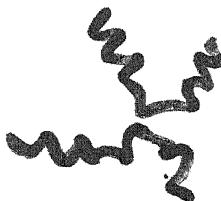
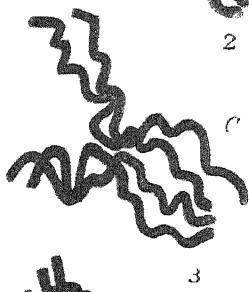
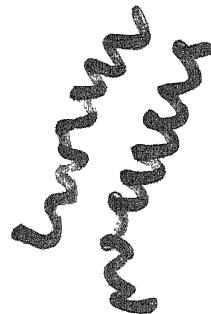


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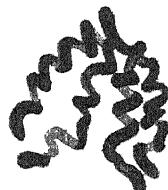
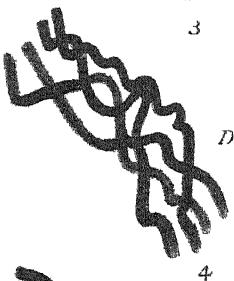
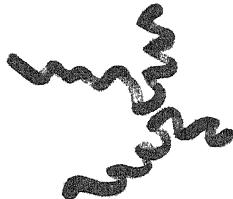
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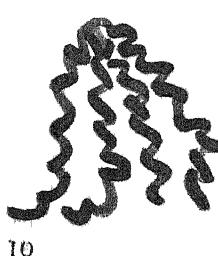
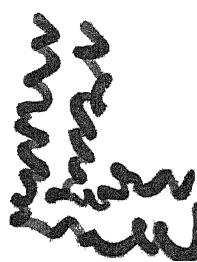
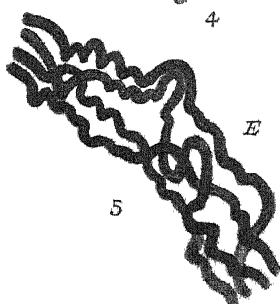
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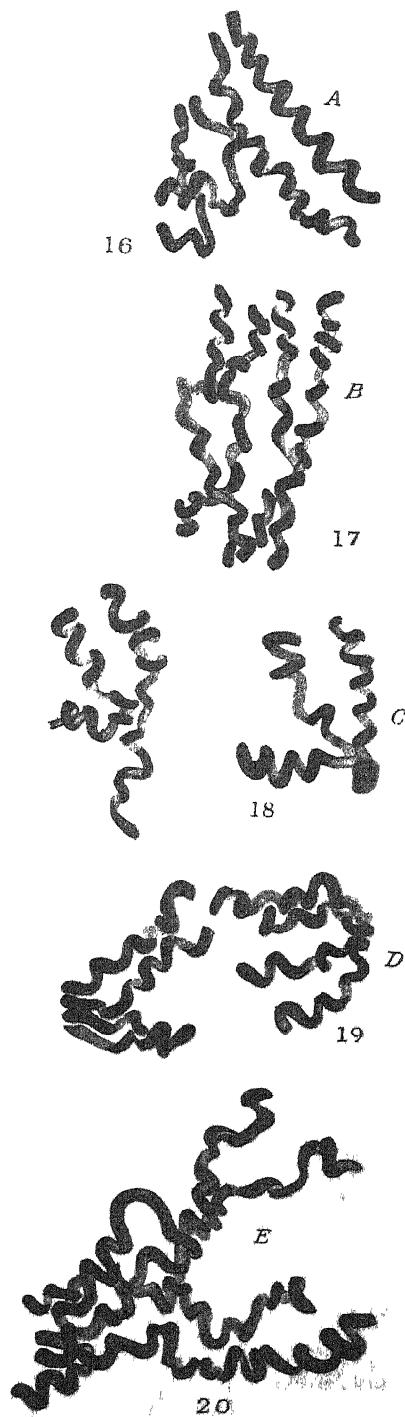
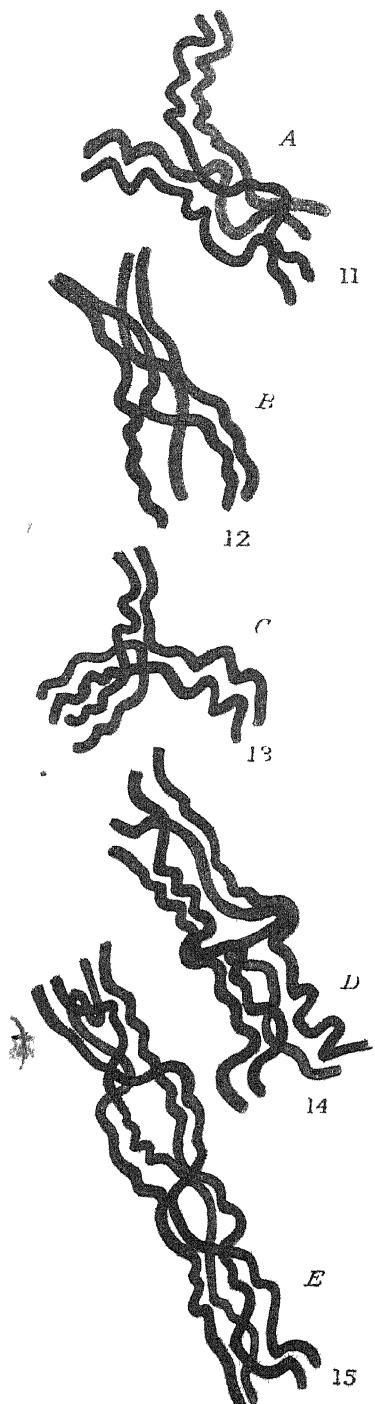


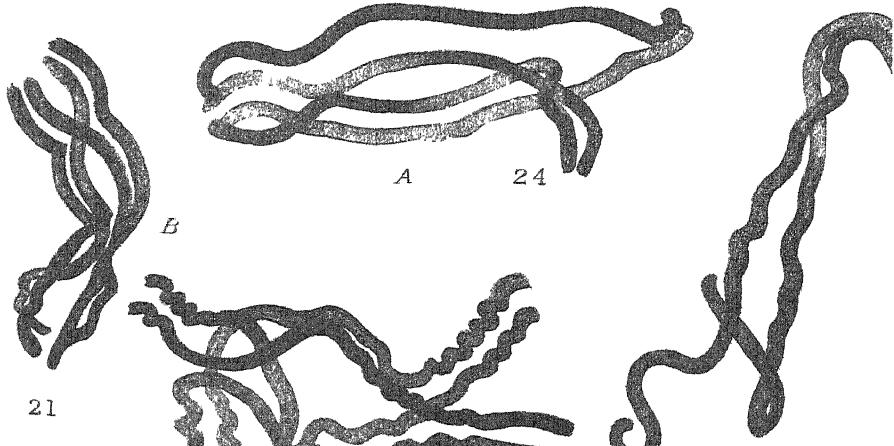
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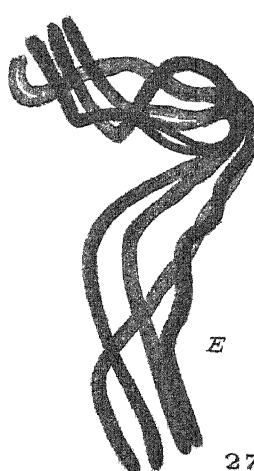
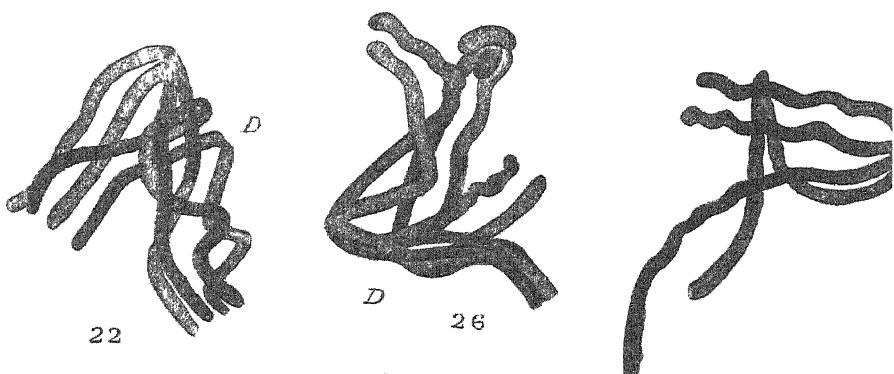
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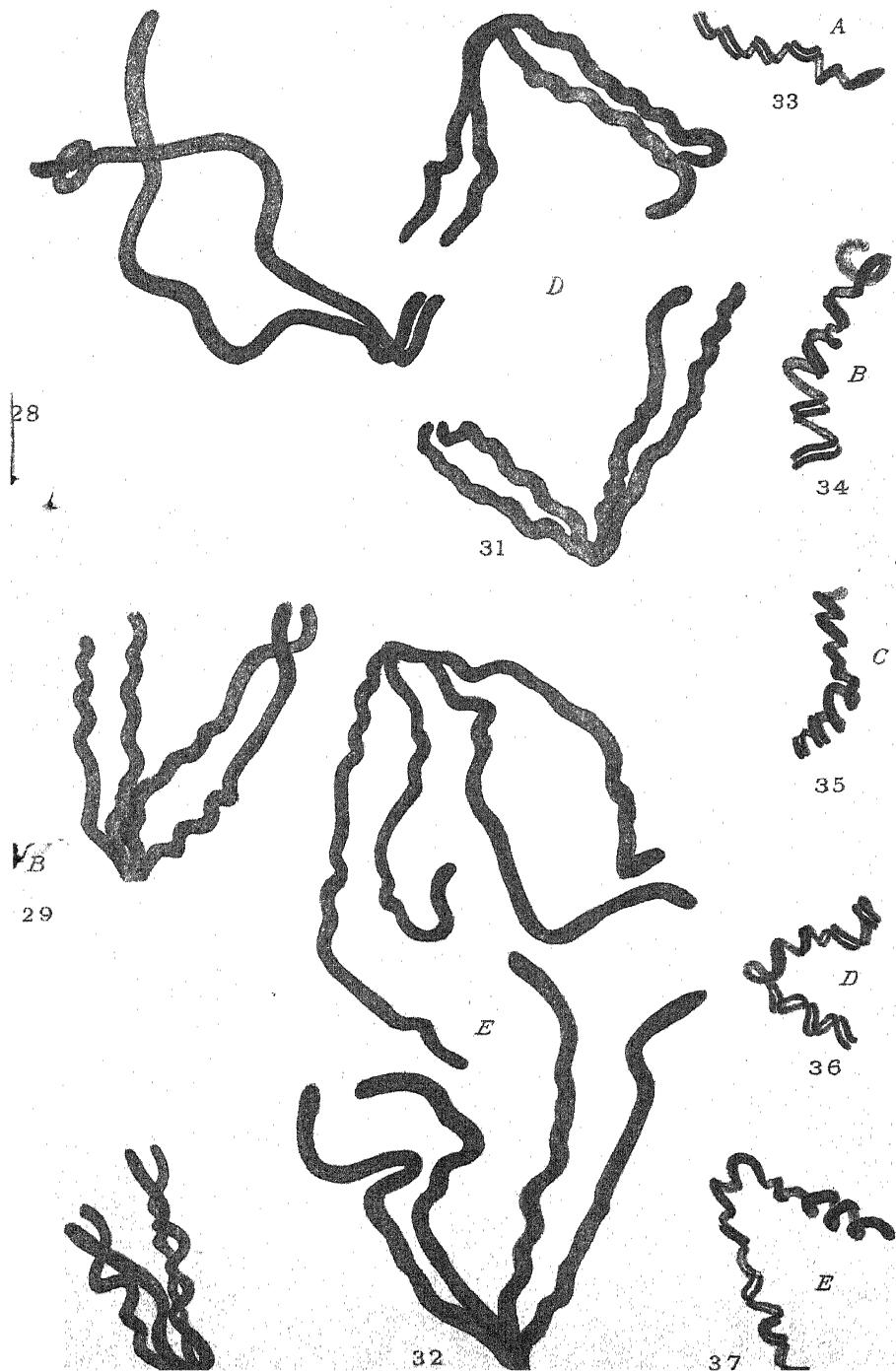


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Cytological Investigations on the Genus Cicer¹

BY

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With Plate VII and eighty-seven Figures in the Text

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I. INTRODUCTION

CICER ARIETINUM Linn. is one of the important pulse crops of India, where it is familiarly known as 'Bengal Gram' or simply as 'gram'. In England it is also known as 'Chick pea'. This plant was known to the Greeks in Homer's time under the name 'Erebinthus' and to the Romans as 'Cicer'. The botanical specific name owes its origin to a not altogether fanciful resemblance of the seed when first forming in the pod to a ram's head (the 'krios' of the Greeks). Watt (1908) believes that it is indigenous to the south-east of Europe. He thinks that it must have been introduced very early into India by the Western Aryans, as it bears a Sanskrit name. No records have been found of the cultivation of this crop in China, Japan, Australia, or America. Kawakami (1930) makes no mention of the plant in his list of chromosome numbers of Leguminosae. Its cultivation in Russia can be inferred by the works of Dombrowsky-Sludsky (1927) and others. Guignard (1881), in his studies on the morphology of Leguminosae, refers to the peculiarities in the

¹ Part I of thesis approved for the M.Sc. degree in the University of London.

[Annals of Botany, N.S. Vol. III, No. 10, April 1939.]

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opment of the embryo-sac. The seeds of this plant are sold throughout the world.

This crop exhibits marked phenotypic variations in seed-coat colour, flower colour, habit of the plant, &c. In India Howard, Howard, and Khan (1915) described the various varieties. Shaw and Khan (1931) and others have studied the inheritance of various characters. Very little attention has been given to the cytology of this plant. The purpose of the present paper is to achieve this object.

II. CLASSIFICATION

Bentham and Hooker in 'Genera Plantarum', vol. i, place this plant under the sub-order Papilionaceae of Leguminosae and in the tribe Vicieae. In this tribe the following six genera have been included:

Tribe, VII. *Vicieae*. *Cicer*.
 Vicia.
 Lens.
 Lathyrus.
 Pisum.
 Abrus.

Cytologically, much attention has been paid in various ways to the genera, *Cicer* (Sharp, 1914; Sakamura, 1914, 1915; Heitz, 1926, 1931; Sveshnikova, 1927, 1929; and others); *Lathyrus* (Latter, 1926; Maeda, 1928, 1930; Upcott, 1937; and others); and *Pisum* (Cannon, 1903; Strasburger, 1910; Marshak, 1931; Hakansson, 1929, 1931, 1932; and others). The lentil has received the name *Lens esculenta* Moench, *Cicer lens* Willd., and *Ervum lens* Linn. Thus, there is great confusion in its nomenclature. Its chromosome number has been reported to be $2n = 14$ (Sakamura, 1920; Heitz, 1926; Bleier, 1928; Miranda, 1931). The cytological investigations of this plant will be published in another paper. The somatic number of *Abrus precatorius* is 22 (Chekhov).

In the genus *Cicer* a large number of species have been reported in Index Kewensis. These can be classified roughly into twenty-two species. They are all distributed in parts of Asia Minor and Egypt, where they occur only in the wild state. One species, *C. soongaricum*, is cultivated in parts of the Western Himalayas. The grains of this plant are said to fatten cattle quickly and the young shoots are used as pickles by the Chinese and a vinegar is made from the leaves. The seeds of two other wild species, viz. *C. pinnatifidum* Jaub. & Spach. ($2n = 16$, Avdulov, 1937) and *C. montbretii* Jaub. & Spach., are extremely small. The seeds of the former are triangular and have tiny spines on the seed-coat. In the latter, the seeds are round and the seed-coat is smooth. Unfortunately, these seeds did not germinate in spite of all treatments.

III. PREVIOUS WORK

The mitosis of *C. arietinum* was first studied by Dombrowsky-Sludsky (1927), who found the $2n$ number of this species to be 14. Rao (1929) found the same number. Dixit (1932a), next reported that the 'desi' type of *Cicer* with small brown seeds had 14, while the 'Kabuli' varieties (which he called *C. Kabulium*) with large white seeds had 16 chromosomes. Further (1932b), in another paper he said that a 'desi' type (Pusa type no. 22) having 14 chromosomes gave rise to a mutant having 16 chromosomes, which he called *C. gigas*. Tischler's list (1935-6) gives the number reported by Milovidov (1932) as $2n = 16$, and also the numbers of Dixit (1932 a). Hruby (1932) quotes the work of Milovidov (1932), who found 16 chromosomes in the root-tips of *C. arietinum* and in mixoploid tissue cells with 32 chromosomes. Avdulov (1937) found that both *C. arietinum* and *C. pinnatifidum* had 16 chromosomes, the chromosomes of the latter being bigger. According to Chekhov, the $2n$ number of both *C. arietinum* and *C. pinnatifidum* is 16.

IV. MATERIAL AND METHODS

I am indebted to the following for the supply of seeds:

A. (a) *C. arietinum* Linn.

1. Director of Agriculture, Imperial Agricultural Research Institute, Delhi, India.
2. Deputy Director of Agriculture, Poona, India.
3. The Cotton Specialist, Agricultural Research Institute, Coimbatore, India.
4. Deputy Director of Agriculture, Quetta, Baluchistan.
5. Mr. N. Krishnaswamy, Botanical Institute, Kiel, Germany.

(b) *C. soongaricum*, Steph.: Director of Agriculture, Kashmir, India.

(c) *C. pinnatifidum*, Jaub. & Spach.: Mr. N. Krishnaswamy, Botanical Institute, Kiel, Germany.

(d) *C. Montbretii*, Jaub. & Spach.: Mr. N. Krishnaswamy, Botanical Institute, Kiel, Germany.

Samples of *C. arietinum* purchased in the London market were also examined.

The seeds were sown in the greenhouse at the Courtauld Genetical Laboratory, Regent's Park, London, in the first week of March 1937. It is interesting to note that *C. arietinum* thrives well in London in the greenhouse and comes to flower in about two months. The varieties exhibited marked variations in the vegetative and floral characters. The seeds of *Cicer pinnatifidum* and *C. Montbretii* offered great difficulty for germination.

Satisfactory somatic metaphase plates were obtained with the following fixatives: 2BE; Navashin's chromic-acetic formalin and Levitsky's chromic formalin. For flower buds, prefixation in Carnoy (Semmens 1937) for 30-40

seconds and fixing in Navashin's solution gave, in general, satisfactory results. The root-tips were fixed between 10 a.m. and 3 p.m. The division periods varied, depending upon the conditions of the weather. The divisions were not simultaneous in all the anthers of a flower, while some were in the first division others were already in the tetrad stage. The root-tips were cut from 10–20 μ and the flower buds from 8–12 μ . After osmic fixation, bleaching was always done by treating the slides in 3 parts 80 per cent. alcohol and 1 part H₂O₂. In certain cases the osmic acid was substituted by uranic acid (Semmens, 1937), and in this case bleaching was hastened by adding a pinch of lithium carbonate to the bleaching solution, keeping the jar containing the slides on a warm plate from 45 to 50 minutes. The stain used was gentian violet after mordanting with iodine in 80 per cent. alcohol. In the flower buds the cytoplasm was often stained heavily, and this was overcome by treating the slide before staining in 2 per cent. acetic acid for about half an hour and washing in water for one hour (Sammens., unpub.). The staining of the materials fixed in uranic acid was found to be much brighter than those fixed with osmic.

V. SOMATIC DIVISIONS IN *CICER ARIETINUM* AND *C. SOONGARICUM*

The resting nucleus of both *C. arietinum* and *C. soongaricum* has a single large nucleolus. In the former it always tends to lie to one side of the nucleus, whereas in the latter it is more or less central. In *arietinum* the 'reticulum' is made up of thin achromatic threads, interspersed with masses of chromatin (prochromosomes; Overton, 1905), which are more or less equal to the chromosome number ($2n = 16$). These bodies are best seen after fixation in Navashin's fluid or in Levitsky's solution, already mentioned. The prominent prochromosomes seen in *arietinum* were not found in the resting nuclei of *soongaricum*, in which the chromosomes are considerably larger than in *arietinum* (Text-figs. 1, 2). The question of prochromosomes will be dealt with later.

The prophase starts with the gradual condensation of chromatin along the thread on either side of the prochromosomes. The chromosomes are at first thin and long. Gradually they thicken and coalesce with the prochromosomes. In favourable preparations, especially in *soongaricum*, the primary constrictions are clearly seen. The chromosomes occupy a peripheral position in the nucleus, and size differences could also be made out. In favourable cases four chromosomes are seen attached to the nucleolus in *C. arietinum* (Text-fig. 3). Dombrowsky-Sludsky (1927) says that all chromosomes are at first attached to the nucleolus and later the connexions are lost. Such behaviour was not met with in any case. In the late prophase the split halves of the chromosomes are seen clearly, the degree of separation of the chromatids being more marked in *soongaricum*. In the metaphase plates 16 chromosomes in *arietinum* and 14 in *soongaricum* are clearly seen, the chromosomes of the latter being much bigger. This applies to the whole complement of *soon-*

Syengar—Cytological Investigations on the Genus *Cirsium*.

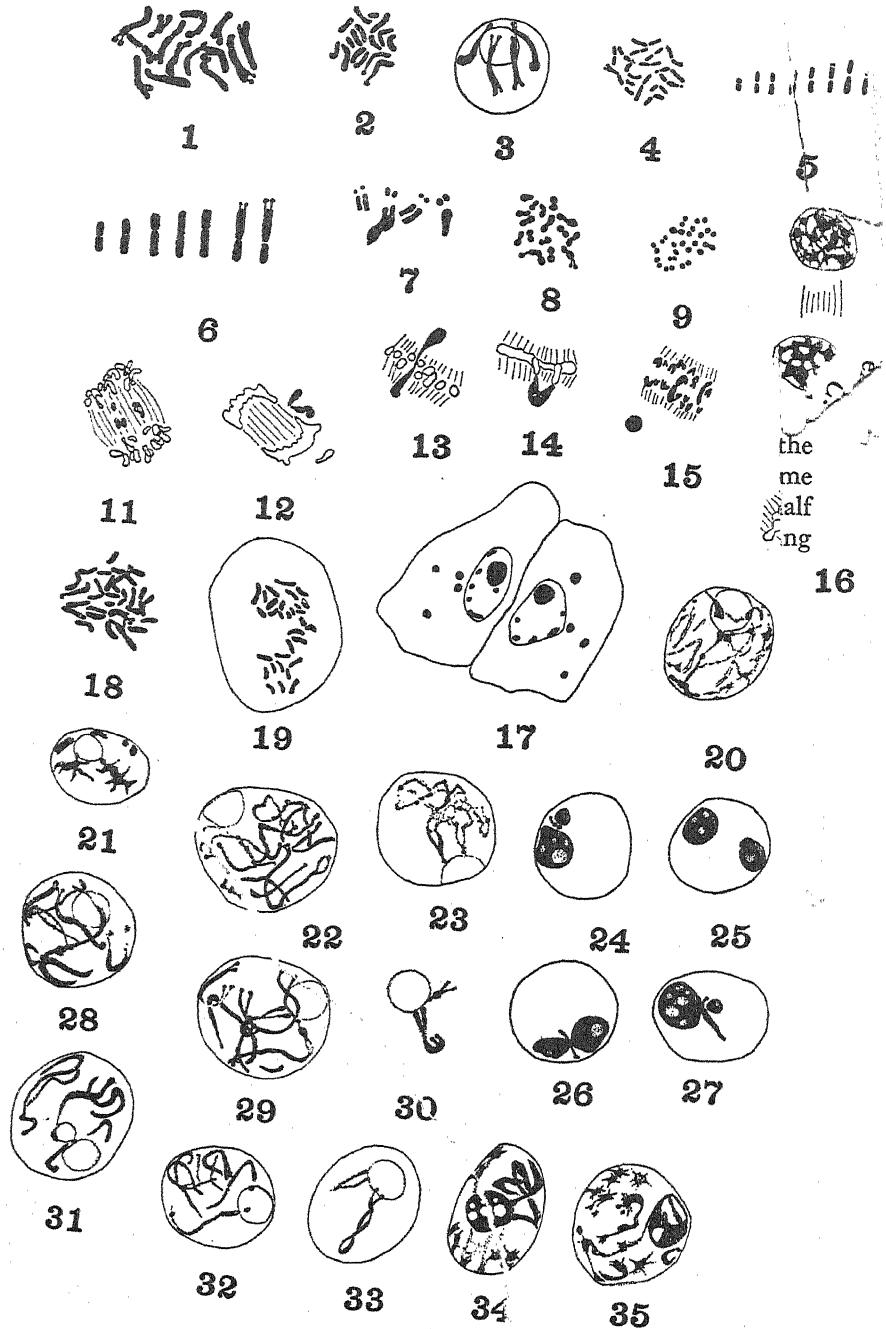
icum, suggesting that it has been brought about by the action of the gene. Avdulov (1937), working with *C. arrietinum* and *C. pinnatifidum*, finds that both species have 16 chromosomes. Comparison of his figures with mine shows that the chromosomes of *C. pinnatifidum* are intermediate in size between those of *C. arrietinum* and *C. soongaricum*. It is interesting to note that cases of the entire complement of chromosomes undergoing either a general increase or decrease in size have been reported in hybrids. Navashin (1934) in *Crepis* hybrids found such an increase or decrease in the hybrids as compared with their parents. Similar cases have been reported by Claussen (1931) in *Viola* and others. The details of the chromosome complement of the two species examined by me and that of *C. pinnatifidum* examined by Avdulov (1937) are as follows.

C. arrietinum ($2n = 16$) (Text-fig. 4). Chromosomes very small with a range of size, the shortest pair being about one-third the length of the longest pair. From Text-fig. 5 it is seen that there is a marked increase from the first to the second pair and another marked increase in the sixth and seventh pair. A similar observation has been made by Avdulov (1932) in this species. The sixth pair has satellites. The complement is made up of one very short pair with median or sub-median constriction; two pairs fairly short but much longer than the first pair, with median or sub-median constriction; three pairs medium, the lengths of which steadily increase from third to sixth with median or sub-median constriction, the longest having a satellite; one pair long with median constriction; one pair long with a satellite and sub-median constriction. Thus there are two pairs of satellite chromosomes of different length.

C. pinnatifidum (taken from Avdulov) ($2n = 16$). The chromosomes are intermediate in size and form a graded series without gaps. The complement is made up of one short pair with satellites and sub-median constriction; one pair short without satellites, with sub-median constriction; five medium pairs which show a steady increase in length, having a median or sub-median constriction; one long pair with median or sub-median constriction.

C. soongaricum ($2n = 14$). Chromosomes much larger than the above (Text-fig. 6). The complement is made up of one very short pair with median or sub-median constriction; one fairly short pair with median or sub-median constriction; three pairs medium with sub-median constriction; two pairs long with sub-terminal constriction, of which one pair has a large satellite and the other a small (Text-fig. 7).

In both the species examined by me the even distribution of the chromosomes on the metaphase plate is modified by the phenomenon of somatic pairing (Text-fig. 2). The homologous chromosomes tend to lie in pairs, as in Diptera (Metz, 1916). A similar tendency is seen in *C. pinnatifidum*. In the case of chromosomes showing size differences the shorter ones occupy the central area. There are, however, exceptions to this rule, as in the case of the tree-cricket (*Oecanthus longicauda*, Makino, 1932), where the short pairs



occupy the peripheral position, and also in certain moth hybrids. In the present case the position of the short pairs varied on the plate (Text-fig. 2, 4).

In plants somatic pairing is met with in both diploids and polyploids. In the latter, as in *Gossypium*, there are indications that four homologous chromosomes may sometimes be paired. Somatic pairing is well seen in those cases where the doubling of the chromosomes has taken place by failure of separation of the daughter chromosomes (Spinacea: Stomps, 1911; Sorghum: Huskins and Smith, 1932; Iberis: Manton, 1935; &c.). In certain instances the pairing is limited to the ends, as in *Oryza* (Kuwada, 1910). In the present case it is usually parallel. Often the small ones pair at the

All figures were drawn at the table level with the aid of a camera lucida. A 2 mm. Zeiss Apochromatic, 1:3 aperture, and Zeiss ocular $\times 20$ were employed for all drawings, excepting for Figs. 63 and 65. The magnification for all the drawings is $\times 3,500$.

TEXT-FIGS. 1-35. Fig. 1. *C. soongaricum*. Somatic metaphase. Fig. 2. *C. arietinum*. Somatic metaphase. Note the variable position of the short chromosomes on the plate, and somatic pairing. Fig. 3. *C. arietinum*. Somatic prophase, showing 4 chromosomes attached to the nucleolus. Fig. 4. *C. arietinum*. Somatic metaphase showing 16 chromosomes and constrictions. Fig. 5. *C. arietinum*. Idiogram. Note the sudden increase in length from the first to second, and another increase from the 6th to 7th. Satellites are seen on the 6th and 8th chromosomes. Fig. 6. *C. soongaricum*. Idiogram. Note satellites on chromosomes 6 and 7, and a steady increase in length from the shortest to the longest chromosome. Fig. 7. *C. soongaricum*. Metaphase chromosomes showing 2 big and 2 small satellites. Fig. 8. *C. arietinum*. Somatic anaphase. Only one plate shown. Fig. 9. *C. arietinum*. Polar view of somatic anaphase showing the marked shortening of the limbs and the gap between the paired bodies representing the spindle attachment region. Fig. 10. *C. arietinum*. Partly optical section of a polar view of somatic telophase. Figs. 11 and 12. *C. arietinum*. Showing lagging chromosomes in somatic anaphase. Figs. 13-17. *C. arietinum*. Stages showing the behaviour of the persisting nucleolus when caught in the metaphase plate of the somatic division. Fig. 13. Nucleolus caught in the metaphase plate and leading to equal division on either side of the plate. Fig. 14. Nucleolus held by the chromosomes at metaphase. Fig. 15. Early anaphase, the nucleolus at one pole only, far in advance of the chromosomes. Fig. 16. Unequal distribution of the nucleolus to the two poles. Fig. 17. Interphase nucleolus lost in the cytoplasm. Figs. 18 and 19. *C. arietinum*. Somatic metaphase plates showing 32 chromosomes. Fig. 20. *C. arietinum*. Leptotene stage showing the attachment of 4 chromosomes to the nucleolus. Note that the region of attachment is more chromatic and the knobs double. Partly optical. Fig. 21. *C. arietinum*. 4 prochromosomes attached to the nucleolus in a premeiotic pollen mother cell. The remaining prochromosomes are not shown. Fig. 22. *C. arietinum*. Late zygotene (partly optical). Fig. 23. *C. arietinum*. Synizesis stage, showing the attachments of two bivalents to the nucleolus. Figs. 24, 26, and 27. *C. arietinum*. Zygote stage. Nucleolus with a bud and a chromosome between the two (threads are not shown). Fig. 25. *C. arietinum*. Zygote stage, with a bud at the opposite end of the nucleus (threads not shown). Fig. 28. *C. arietinum*. Late pachytene stage, and the beginning of diplotene in some of the bivalents. Fig. 29. *C. arietinum*. Pachytene, showing all the eight bivalents. Two bivalents are attached to the nucleolus. Fig. 30. *C. arietinum*. Showing attachment of two bivalents to the nucleolus. In one bivalent the pairing is hindered at the region of the attachment. Fig. 31. *C. arietinum*. Pachytene. Two bivalents attached to the nucleolus. Of these one is between the nucleolus and the bud, and the other attached to the bud. Fig. 32. *C. arietinum*. Pachytene showing the attachment of two bivalents to the nucleolus. Fig. 33. *C. arietinum*. Pachytene showing the attachment of two bivalents to the nucleolus. The pairing at the region of the attachment is hindered in both the bivalents. Figs. 34-5. *C. arietinum*. Various stages of diffusion at diplotene. All the figures show only the optical section. Attachment of the chromosomes to the nucleolus is seen in Figs. 34 and 35.

end only, thus giving a false V-shaped chromosome. Such V's have been regarded by some as a single chromosome, in *C. arietinum*, thus making out the diploid number to be fourteen. Such pairing at the ends indicates an end homology, and as will be shown later, there are indications that segmental interchange has played a part in the establishment of the apparently diploid genome of *C. arietinum*. Gates (1911 a) in *Oenothera* takes the view that this paired condition between homologous chromosomes may occur throughout the sporophyte. Koller (1934) assumes that somatic pairing may be conditioned by the genotype controlling the degree of attraction between homologous chromosomes.

In *C. arietinum* at anaphase the chromosomes with sub-median centromeres show the longer arm projecting from the 'tassemont polaire'. The polar view of anaphase shows the dumb-bell-shaped chromosomes caused by the marked shortening of the limbs on either side of the spindle attachment region (Text-fig. 8). Such dumb-bell-shaped chromosomes are seen in *Oenothera* (Gates, 1912), *Trigonella* (Fryer, 1930), &c. At later stages the limbs continue to contract, and in polar views are seen only as small paired bodies, the gaps in the middle being the spindle attachment region (Text-fig. 9).

At telophase, excepting for the regions on either side of the spindle attachment which are so close to each other as to appear as a single body, the other regions of the chromosomes become completely achromatic (Text-fig. 10). The origin of the nucleoli is hard to make out. At slightly later stages several nucleoli are seen, but they cannot always be distinguished from prochromosomes. They gradually fuse, forming four, three, or two bodies. These may further fuse into a single nucleolus characteristic of the resting nucleus. In *C. soongaricum* three nucleoli are very common and the maximum number is four. These in the early telophase correspond with the four satellites present on the metaphase plate.

Somatic lagging. Changes in somatic mitosis may be due to non-disjunction in which a cell with a new number ($2n+1$) or ($2n-1$) may arise, as in *Datura* (Blakeslee and Belling, 1924), where shoots with ($2n-1$) and ($2n+1$) chromosomes were found, and these latter are perpetuated. Similarly, De Mol (1927) in *Tulip Murillo* ($2n = 24$) found that one chromosome was sometimes missing. A similar case happened in triploid *Hyacinthus* ($3n = 24$), where only 23 somatic chromosomes were found. According to De Mol (1927), dwarf *Hyacinthus* with 18 and 21 chromosomes arose by somatic non-disjunction being stimulated by injury to the bulbs. Variation in number can also arise by non-division.

Besides these methods, the variation in the chromosome number of the daughter cells during mitosis may also be caused by the lagging of chromosomes. In *C. arietinum* three instances of lagging were noted in somatic divisions (Text-figs. 11, 12). If such behaviour takes place in the premeiotic division a varying number of bivalents would be seen in the first metaphase.

Such irregular numbers were found in this material, as described later. Somatic lagging has been reported in the sixth generation of the hybrid between wheat and rye (Plotnikowa, 1932). The percentage of abnormal mitosis varied from 0 to 33 per cent. in the plants studied. Olmo (1937), working with *Vitis vinifera*, reports that in somatic divisions at metaphase the chromosomes failed to align on the plate. Somatic lagging has also been reported by Crew and Koller (1932) in the mouse. Here the chromosomes were sometimes held together in pairs, the distal ends remaining in association. Such terminal association was seen in Text-fig. 12.

Nucleolar fragmentation. In the resting stage the nucleolus is more or less spherical. During prophase it becomes irregular in outline, as seen by Wager (1904) in *Phaseolus* and by Latter (1926) in *Lathyrus*. It gets considerably reduced in size and by metaphase is completely lost. Very frequently it persists, as seen by Zirkle (1928) in *Zea*, Frew and Bowen (1929) in *Cucurbita*, and others. In the present investigation persistence of the nucleolus is also seen in the first metaphase and also in pollen-grain divisions. The behaviour of the persistent nucleolus during mitosis is very similar to the case reported by Frew and Bowen (1929) in *Cucurbita*. When caught in the metaphase plate it elongates in the direction of the length of the spindle. Later, the two halves on either side of the plate are stretched and the nucleolus constricts into two, which migrate to the respective poles in advance of the daughter chromosomes. The length abstracted on either side of the equator depends upon the region of the nucleolus caught in the plate. Sometimes the whole nucleolus passes to one side only. In Text-figs. 13–17 the various stages are shown. The nucleoli ultimately reach the pole, fragment and are lost in the cytoplasm (Text-fig. 17).

Chromosome numbers. In *C. arietinum* a large number of varieties have been isolated. As already stated, extreme phenotypic variations in height, branching, size of pods and seeds, shape of the seeds (Pl. VII, Fig. 1), and other characters, are met with in this crop. Thirty varieties collected from various places were examined for chromosome numbers. In all cases the number was found to be 16. Milovidov (1932), Avdulov (1937), also report that all the varieties examined by them had 16 chromosomes. Also no differences were seen in the size of the chromosomes in the varieties. In *soongaricum* also, of the two varieties examined, whose chromosome numbers were found to be 14, there was marked phenotypic variation in leaf-shape, shape of the seed, &c. A similar constancy in the number of chromosomes is seen in the genera such as *Lathyrus* and *Pisum*. Only in *Vicia* is there marked variation number, the haploid numbers being 5, 6, 7, 12, and 14 (Heitz, 1926, 1931; shnikova, 1927, and others).

Tetraploidy. In *Cicer arietinum* occasional chimeral areas were met with containing 32 chromosomes (Text-fig. 18, 19). These areas were found in periblem region of the root and in the same section diploid numbers were seen. Such cases of mixoploidy have also been reported by Milovidov

(1932) in this species. Cases of somatic doubling in the root-tips have been reported in a number of plants, and in some the modified region may be a well-defined sector of the plant; as *Crepis* (Navashin and Hollingshed, 1926, 1930), *Lycopersicum* (Lesley, 1925), *Nicotiana* (Ruttle, 1928), &c. Langlet (1927) lists known cases. Hruby (1932) and Meurman (1933) give a full review of this subject.

Tetraploidy in somatic cells may be caused by failure of cross-wall formation between the daughter nuclei, thus resulting in a binucleate cell. By later fusion or by simultaneous division of these nuclei, tetraploid numbers may arise. This condition is indicated in Text-fig. 19. The chromosomes may also split and double their number without division of the nucleus. If such an aberration occurs in a zygote or in a young embryo, a tetraploid individual would arise directly, as in *Oenothera* (Gates 1909, 1913, 1924), *Triticum* (Sax 1921, 1922), &c. Winge (1917) attributes such doubling to chromosomal incompatibility. Randolph (1932) obtained tetraploid plants in *Zea* by artificially heating young ears soon after fertilization. According to de Litardiere (1923), in root-tips of *Spinacea* the chromosome halves may separate during prophase and undergo a second splitting, so that each telophase nucleus receives a tetraploid number rather than a diploid number.

Somatic doubling in plants can be induced by external agencies, as action of heat (Randolph, 1932), in the callous tissue caused by injury (grafting in tomato by Winkler, 1916), and others; inoculation of poisonous substances or bacteria (De Mol, 1931; Nemec, 1910). The formation of tetraploid cells in callous tissue has been taken advantage of by Winkler (1916) and others to get tetraploid shoots in Solanaceae.

In the case of hybrids, somatic doubling is of great significance in making the plants fertile. Thus in a sterile hybrid a tetraploid shoot may arise by somatic doubling by some method (*Primula kewensis*, Digby, 1912; Newton and Pellew, 1929, &c.), and this shoot may be highly fertile, while the rest show marked sterility. Diploid shoots from haploids develop with exceptional vigour, as in *Crepis* (Hollingshed, 1930), *Datura* (Blakeslee and Belling, 1924), &c.

VI. MEIOSIS IN *C. ARIETINUM*

The pollen mother-cells are distinctly polygonal in outline with no spaces between them or between those at the periphery and the tapetum. The space observed is due to contraction of the mother-cells by fixation. The tapetal cells remain uninucleate throughout the divisions of the pollen mother-cells, as in *Lathyrus* (Latter, 1926).

The resting nucleus of the pollen mother-cell presents a faintly stained reticulum with a large deeply staining nucleolus more or less spherical shape. This is always found to be on one side of the nucleus. The edge of the nucleolus against the nuclear membrane is flattened. Distributed in the reticulum are the prochromosomes, which are roughly equal in number.

the chromosomes. A clear area around the nucleolus was not met with in good fixations, and according to McClung (1929) and Derman (1933) it is a distinct artifact caused by fixation.

The nucleolus shows a large vacuole, and sometimes crystal-like bodies were seen in the vacuoles. Such bodies in the vacuoles have been reported in *Oenothera franciscana* (Cleland, 1922, 1924), *Galtonia candicans* (Digby, 1909), *Lathyrus odoratus* (Latter, 1926), &c. Their function is not clearly known.

Prophase of the first division. Owing to the small size of the chromosomes and the delicate nature of the threads the details of prophase cannot be fully interpreted. This stage begins with an increase in size of the nucleus. A clear leptotene stage is seen. The threads are thin, long, and beaded (Text-fig. 20), but too fine to determine their nature.

The existence of a leptotene stage in nuclei showing prochromosomes is disputed. Lundegårdh (1909) found that although the prochromosomes are arranged side by side during early prophase they do not conjugate until the time of synzetic contraction. De Souza (1929) stated that univalent leptotene strands do not exist in *Impatiens balsamina*. Leliveld (1932) in *Oenothera franciscana* concluded that prochromosomes tend to pair in the earliest prophases but that some univalent strands are present after synizesis. Smith (1934) in *Impatiens balsamina* says that the leptotene strands do exist. Marquardt (1937) in *Oenothera hookeri* finds prochromosomes and a clear leptotene stage.

In the present material leptotene threads are seen and four of them are attached to the nucleolus (Text-fig. 20), in agreement with the attachment of four chromosomes in somatic prophase and four prochromosomes to the nucleolus in the resting nuclei (Text-fig. 21). In rice, Nandi (1937) found two chromosomes attached to the nucleolus in one variety and four in another (1936), and in somatic metaphase he found two satellites in the former and four in the latter. He found the knobs attached to the nucleolus double, indicating that the threads are already double. In the present material also such doubleness was seen (Text-fig. 20).

Zygotene. The threads begin to pair side by side. They are thin and long and still show the beaded appearance, but are unpolarized (Text-fig. 22). The relationship of the chromosomes to the nucleolus at this time is interesting. In those cases where pairing is complete only two are attached, each representing a bivalent. In such cases the threads at the region of the nucleolus must have been very near to each other, making the complete pairing impossible. In certain cases, the nucleolus seems to offer an obstacle to pairing, resulting in three or four separate attachments. Farther away from the nucleolus the threads are seen paired. In certain cases the reverse was also (Text-fig. 23). In this case the pairing has started from the ends, the fibers of the pair situated near each other at the region of the nucleolus making the pairing at this region possible.

At later stages the threads seem to undergo the characteristic synizesis stage, which according to Professor Gates (quoted from Nandi, 1937) represents a sensitive condition of the nucleus when the delicate threads are easily compacted together by the entrance of the fixing fluid. Thus, this is an artifact.

Another interesting feature of the nucleolus at this stage is the appearance on it of protuberances or globules (Text-figs. 24-7). In most cases only one globule was seen on a nucleolus. The nucleolar buds may be of different sizes and may be either attached to the main nucleolus or far from it (Text-fig. 25). When it is near the main nucleolus a thread is invariably seen to pass between the two. At first the main nucleolus is deeply stained and the bud is practically colourless. Later, the staining capacity is seen to be more in the buds, when they take up a deep stain and the main nucleolus retains very little of it. It looks as though all the stainable material has passed from the main nucleolus to the 'secondary nucleolus', as Selim (1930) calls these buds.

The phenomenon of nucleolar budding has been described in many plants. It seems to appear only in stages from zygotene onwards, but is at present not well understood. It cannot be an artifact of fixation, which Nandi (1937) believes to be the case in rice. The view that these are not buds but stages of the fusion of two nucleoli formed in the previous telophase does not appear to hold good in this material: first, where two nucleoli are not seen in the earlier stages of prophase; secondly, buds appear to be small at first and grow larger. In the light of these difficulties it is unsafe to attach any significance to these buds until more is known about their origin and nature.

Pachytene. The threads are distinctly thicker and shorter but are still too long to make out easily all the bivalents. In the clearest cases eight bivalents could be counted (Text-figs. 28, 29). Two bivalents are distinctly attached to the nucleolus (Text-figs. 30-3). In some the failure of pairing at the attachment region is still seen. Probably no pairing takes place at this region in such cases. The nucleolus moves more to the centre and is spherical. Occasionally nucleolar buds are seen, with a thread passing between the two (Text-fig. 31).

At late pachytene the threads are distinctly thicker and shorter and they begin to repel one another. The four chromatids of each bivalent exchange partners along their length, the points of intersection representing the chiasmata.

Diffuse stage. The beginning of the repulsion marks the diplotene stage. In the present material when repulsion starts in some of the bivalents the nucleus undergoes a remarkable change. This is the 'diffuse stage'. The condition of the nucleus is most unusual among plants, but is of common occurrence in animals. Among plants, it has been reported in *Padina* (Car 1927), *Hyacinthus* (Darlington, 1929a), *Tradescantia* (Darlington, 1929), *Rumex* (Fikry, 1930), *Mitrastemon* (Matsuura, 1935a), &c. Matsuura (1935)

gives a detailed description of the 'diffuse stage' in *Mitrostemon* as follows: The spiremes elongate considerably, take vague contours and become entirely oxyphilic, closely approaching the condition of the resting nucleus. No correlation was found between the occurrence of the nuclear diffusion and the increase of cytoplasmic volume. At this stage the nuclear membrane may be sometimes entirely lost, giving rise to the formation of functionless giant pollen grains. A similar phenomenon was also seen in the embryo-sac mother-cell.

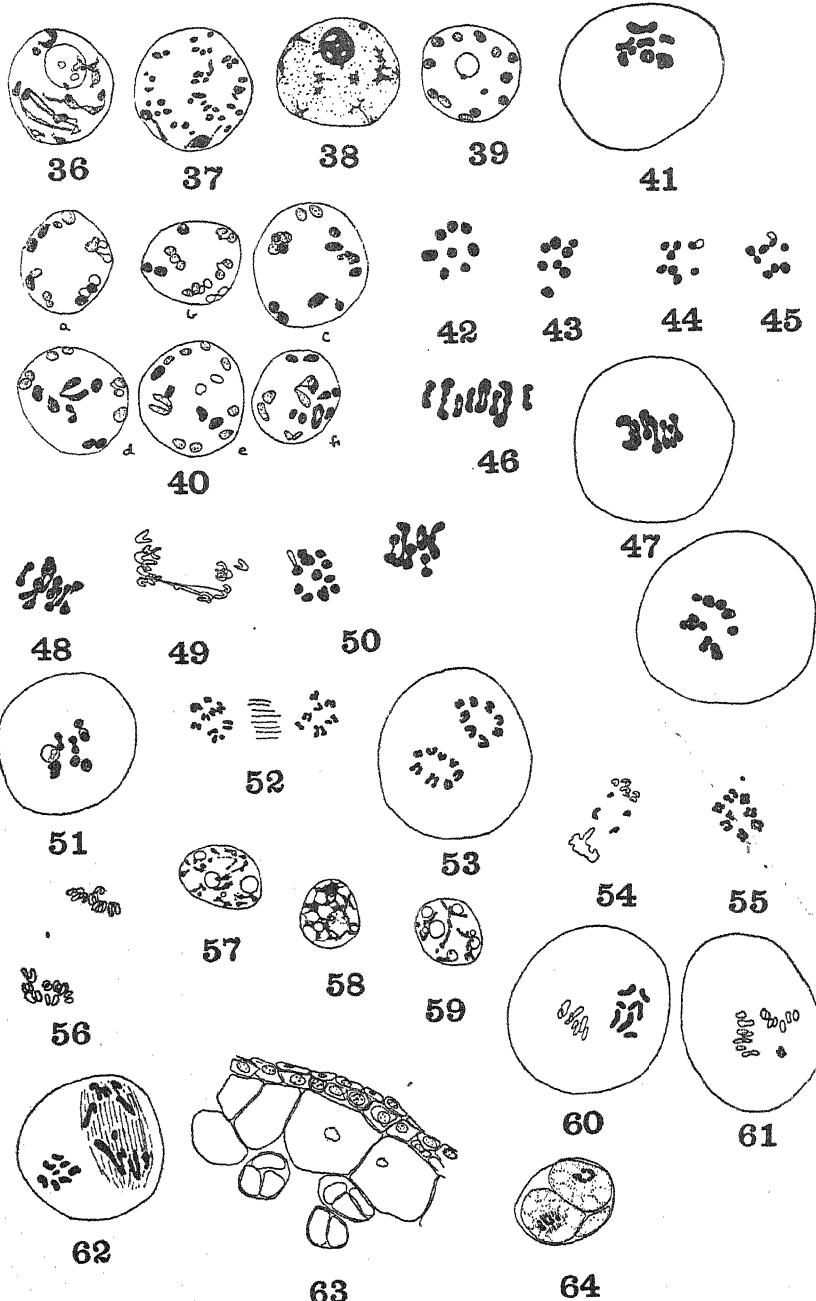
Wilson (1928) classifies the different types of diffuse stage under three heads and is of opinion that this stage is undoubtedly correlated with cytoplasmic growth, for in general the longer the growth period and greater the growth of the auxocytes the greater the nuclear diffusion. He regards this stage as a highly modified diplotene stage in which the duality of the early diplotene, however it may be obscured, may persist throughout.

The diffusion may be of the following kinds. (1) The chromosomes swell and continue the normal course of development in their bloated condition, as in *Pristiurus* (Ruckert, 1892). (2) The chromosomes disappear for a while and reappear again unchanged, as in *Mitrostemon* (Matsuura, 1935a). (3) A part of the nucleus containing the chromosomes condense to one side to form a 'karyosphere' as in *Phanaeus* (Hayden, 1925), in which the condensed pachytene chromosome can be separately distinguished.

In the present case, visible chromosome individuality is practically lost by the loss of chromaticity and sometimes branch-like processes are seen (Text-figs. 34, 35, 38, and Pl. VII, Figs. 2-4). In certain cases the paired condition of the diplotene threads is still seen (Text-fig. 36). In nuclei where the diffusion has reached the extreme limit the threads are entirely lost to view and only chromatin masses are seen, some of which are in pairs (Text-figs. 37, 38). In certain nuclei the relationship of the bivalents to the nucleolus is still maintained (Text-figs. 34, 35). The nucleus was slightly larger, but as seen by Matsuura (1935) in *Mitrastemon* there was no increase in the cytoplasm. After the diffusion, the chromaticity gradually increases and the bivalents are once more visible at diakinesis and fully contracted (Text-figs. 39, 40).

Diakinesis. In early diakinesis, owing to marked repulsion the bivalents are seen arranged at the periphery of the nucleus (Text-fig. 39). At later stages the bivalents are in the form of V, X, O, or rods (Text-fig. 40). In most of the nuclei eight bivalents were seen, very rarely a trivalent and univalent (Text-fig. 40b). A particular bivalent was seen to pair only at one end. The nucleolus is now very much reduced, and two bivalents were seen near it. At mid-diakinesis maximum repulsion is seen. Later, the repulsion weakens and the pairing of the bivalents commences. In late diakinesis the nucleus is considerably reduced in size.

Prometaphase. The nuclear membrane now disappears and the spindle appears in the region of the nucleus. The chromosomes become grouped at the centre (Text-fig. 41). At this stage in polypoids the homologous bivalents



which have failed to pair at zygotene have a chance to associate if they come within one another's sphere of attraction. These associated bivalents remain in juxtaposition until they are again dispersed at the next repulsion phase, which in many plants is interkinesis, but if this stage is very short the association persists up to second division.

Metaphase I. After prometaphase, owing to surface repulsion the single bivalents, and the associated bivalents behaving as a unit, are repelled and distributed on the plate. In the same nuclei, both primary and secondary association may occur. The question of secondary association will be dealt with later.

Polar view of metaphase I shows eight bivalents (Text-figs. 42, 43, 44, 45). In the plates in which secondary pairing is not seen the bivalents assume an arrangement similar to that of floating magnets in an electric field. According to Mayer (1879), when the number of floating magnets is eight, seven are found in the periphery and one in the centre (Text-fig. 42). The other arrangement seen in the present case is six in the periphery and two in the centre. According to Kuwada (1929) the former arrangement is more stable. Similar stable arrangements are seen in *Dicentra spectabilis* (Matsuura, 1935b) and *Campanula persicifolia* (Darlington, 1937), which have eight bivalents. According to the former and Nandi (1936) in rice, the associated bivalents behave as a unit and the arrangement of these groups of chromosomes on the metaphase plate will be according to the floating-magnet principle

TEXT-FIGS. 36-64. Figs. 36-8. *C. arietinum*. Various stages of diffusion at diplotene. All the figures show only the optical section. Note the extreme diffusion in Fig. 38. Note also the precocious condensation of some of the bivalents in Fig. 36. Fig. 39. *C. arietinum*. Early diakinesis. Fig. 40. *C. arietinum*. Diakinesis, showing the various types of bivalents. (b) $6_n + 1_m + 1_r$. (c) 2 bivalents attached to the nucleolus. (d) seven bivalents and two univalents. Fig. 41. *C. arietinum*. Prometaphase. Fig. 42. *C. arietinum*. Metaphase I, showing 7 bivalents at the periphery and one in the centre. Figs. 43-5. *C. arietinum*. Metaphase I, showing secondary association. Fig. 43. 3 (2)+2 (1). Fig. 44. 3 (1)+1 (2)+3 (1). Fig. 45. 1 (3)+2 (2)+1 (1). Maximum association. Fig. 46. *C. arietinum*. Metaphase I. Side view showing ring and rod bivalents. Fig. 47. *C. arietinum*. Metaphase I, showing 9 bivalents in one nucleus and 7 in the adjacent nucleus. Fig. 48. *C. arietinum*. Metaphase I, showing association of ring and rod bivalents. Fig. 49. *C. arietinum*. Anaphase I. A chromatid bridge formed by the persistence of the interstitial chiasma. Fig. 50. *C. arietinum*. Metaphase I, showing 10 bivalents in one nucleus and 6 in the adjacent nucleus. Fig. 51. *C. arietinum*. Polar view of metaphase I showing the nucleolus persisting. Fig. 52. *C. arietinum*. Anaphase I. 7 chromosomes at one pole and 9 at the other. Fig. 53. *C. arietinum*. Anaphase I. Showing chromosomes arranged regularly in a ring in both the poles. Fig. 54. *C. arietinum*. Anaphase I showing the long arm of the chromosomes projecting from the 'tassemant polaire' and lagging univalents. Figs. 55 and 56. *C. arietinum*. Anaphase I showing fragments. Fig. 57. *C. arietinum*. Telophase I. The chromosomes could still be made out though a greater portion of them have become achromatic. Three nucleoli are seen. Fig. 58. *C. arietinum*. Telophase I. Anastomoses formed. Four nucleoli are seen. Fig. 59. *C. arietinum*. Early interkinesis or late telophase. Seven nucleoli are seen. Fig. 60. *C. arietinum*. Metaphase II. Note the chromosomes are long and some are paired. Fig. 61. *C. arietinum*. Metaphase II. One chromosome not congressed on the plate. Fig. 62. *C. arietinum*. Anaphase II, showing a lagging univalent. Fig. 63. *C. arietinum*. Abnormal anther showing the elongated tapetum. Fig. 64. *C. arietinum*. Tetrads of Fig. 63. The nuclei have commenced to divide before separation from the mother-cell.

mentioned above. In the present case such behaviour was not seen (Text-fig. 44). Alam (1936) in *Brassica* also reports a similar distortion in the arrangement of the chromosomes where secondary association is seen. Besides secondary pairing, the arrangement is disturbed by non-congregation and non-orientation of the chromosomes, met with frequently in the present material.

The side view of metaphase shows a varying number of rings and rods (Text-fig. 46). Interstitial chiasmata persist in some (Text-figs. 46, 47). Associations of ring bivalents and rod bivalents were seen (Text-fig. 48). In polar view these look like associations of bivalents of different sizes, the rings appearing as big chromosomes and the rods as small ones. Some (Skovsted, 1929) have reported this condition as non-homologous pairing. Recently, Upcott (1936) has convincingly shown in *Aesculus carnea* that such differences in the size of the associated bivalents as seen in polar views are due to differences in the chiasma frequency, the rods having a single terminal chiasma, and the rings two terminal chiasmata. In the rods the anaphase has already started and as a result of tension they are comparatively more attenuated.

In the present material it is the longest and the shortest chromosomes that invariably form the rod bivalents. For in the former the centromere is sub-median and terminalization of the chiasma takes place earlier in the shorter arm, as a result of which the loops separate earlier in the shorter arm, resulting in a rod bivalent. In the case of the shorter chromosomes, which also have a sub-median centromere, a chiasma is formed on only one side. The numbers of rods and rings were counted from twenty metaphase plates. The frequency is as follows:

All rods	1 ring	2 rings	3 rings	4 rings	5 rings	6 rings	7 rings	8 rings
—	7 rods	6 rods	5 rods	4 rods	3 rods	2 rods	1 rod	—
1	3	4	7	4	1	—	—	—

The analysis of the number of chiasmata per bivalent at Metaphase I and the mean number of chiasmata terminalized are as follows:

Number of nuclei	:	:	:	:	:	:	22
Number of bivalents	:	:	:	:	:	:	173
Total number of chiasmata	:	:	:	:	:	:	229
Mean number of chiasmata per bivalent	:	:	:	:	:	:	1.32
Number of terminal chiasmata per bivalent	:	:	:	:	:	:	1.17
Terminalization coefficient	:	:	:	:	:	:	0.89
Highest number of chiasmata in any bivalent	:	:	:	:	:	:	2

The persistence of chiasmata sometimes results in the lagging of bivalents. In one case at anaphase a chromatid bridge was seen (Text-fig. 49) formed by the persistence of the interstitial chiasma. In *Brassica* the lagging of chromosomes by the persistence of the interstitial chiasmata has been reported by Catcheside (1934) and Alam (1936).

Cases of nuclei with more or less than eight bivalents were seen at Metaphase I. In two cases nine bivalents in one nucleus and seven in the adjacent

cell were seen (Text-fig. 47). This must have arisen by premeiotic non-disjunction. Somatic lagging, as described in the earlier part of this paper, may also bring about such an irregularity. In two instances there were ten bivalents in one cell and six in the other (Text-fig. 50). This could also have arisen from the above cause. Cases of premeiotic non-disjunction have been reported in *Oenothera* (Hedayatullah 1932), &c. In the hybrid *O. rubricalyx* × *O. eriensis* a mother-cell having 15 chromosomes was seen adjacent to one having 13 chromosomes. Similar instances of premeiotic non-disjunction have been reported by Gates and Sheffield (1929) in *Oenothera* and by Hoar (1931) in *Hypericum punctatum*. In the present species the increase in the number of bivalents at Metaphase I may also arise by cytomixis, as described later.

Rarely at Metaphase I the nucleolus persists (Text-fig. 51). Such persistence is seen in *Brassica* (Catcheside 1934), where the nucleolus frequently remains through all the stages of the first division.

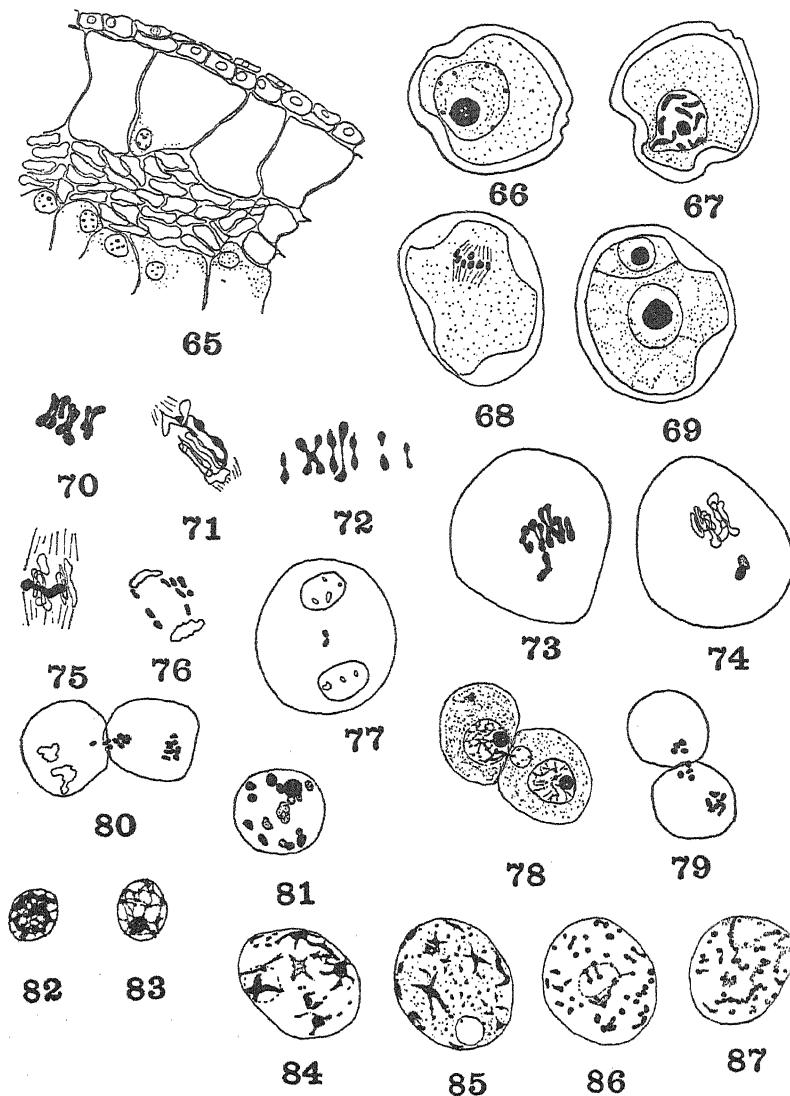
Anaphase I. The anaphase is for the most part regular. The smallest chromosome pair, which contract at metaphase to a short rod, separate as soon as the rest of the complements are arranged on the equator. This precocious separation of small chromosomes seems to depend upon their smaller sizes. In *Ranunculus* such precocious separation of the small bivalents has been reported in certain varieties by Larter (1932). A similar precocious separation is found in the sex chromosomes of *Mus musculus* (Painter, 1927).

Lagging of chromosomes, caused by the formation of univalents or by delay in congression of certain bivalents on to the metaphase plate, was met with. As already stated, lagging is also caused by the persistence of interstitial chiasmata. Hence the even distribution of the chromosomes to the poles is often disturbed. In Text-fig. 52 seven are passing to one pole and nine to the other.

The secondary association seen in metaphase persists in certain cases but generally it vanishes. The chromosomes may be arranged in a ring with either one in the centre and seven peripheral or two in the centre and six peripheral. Sometimes all the chromosomes are arranged in a ring around the periphery of the spindle. In some nuclei this arrangement is symmetrical at the two poles (Text-fig. 53).

At late anaphase the chromosomes are crowded, and in side view the long arm of the chromosomes in which the separation of the chiasma takes place last is still seen projecting from the 'tassemant polaire' (Text-fig. 54). Occasionally small bead-like tips at the end of these projecting chromosomes were seen. It is hard to say whether these are satellites or condensed portions of the arm. Occasionally fragments were seen (Text-figs. 55, 56). These were of different sizes and were found irregularly distributed in the spindle. It is not known how they arose.

Telophase I. The chromosomes become increasingly achromatic (Text-fig. 57). Two to seven nucleoli were seen in daughter nuclei (Text-figs. 57-9). Of the seven one was distinctly larger than the others, indicating



that two have fused. Thus eight nucleoli appear to have been formed in the early telophase in agreement with the eight chromosomes (Text-fig. 59). Such nucleolus-like bodies in relation to all the chromosomes have been reported in several plants; as *Oenothera* (Gates, 1907), *Lathyrus* (Latter, 1926), *Clivia* in somatic telophase (Van Camp, 1924), *Callisia* (Derman, 1933), &c.

Interkinesis. The chromosomes are uniformly distributed as at diakinesis and the dyads held together at the centromere are also seen to repel one another, resulting in X- or V-shaped chromosomes, depending upon the position of the centromere. These show marked repulsion. During interkinesis the chromosomes become elongated (Text-fig. 59).

Division II. The metaphase chromosomes exhibit pairing (Text-fig. 60) and are fairly long, and a satellite could be made out in one of them. Cases of non-congression were met with (Text-fig. 61). The anaphase chromosomes are distinctly in V's. Lagging of chromosomes caused by the univalents was met with (Text-fig. 62).

Though the division is regular, the tetrads were seen to degenerate in a number of cases. In certain abnormal conditions the tapetal cells were enlarged (Text-fig. 63), the pollen grains were not liberated from the mother-cell, and in such cases they showed the first division into vegetative and tube nucleus (Text-fig. 64). Coulter and Chamberlain (1904) quote instances of this in such plants as *Neottia ovata*. The tapetum remains as a single

TEXT-FIGS. 65-87. Fig. 65. *C. arietinum*. Abnormal anther showing degenerated tetrads and elongated tapetum. Figs. 66-9. Various divisions of the pollen grain. Fig. 66. *C. arietinum*. Pollen grain. Nucleus not yet divided. Fig. 67. *C. arietinum*. Prophase of the I division of the pollen grain. Fig. 68. *C. arietinum*. Metaphase of the I division of the pollen grain. Fig. 69. *C. arietinum*. Pollen grain divided into the tube and the generative nuclei. Fig. 70. *C. arietinum*. Side view of Metaphase I, showing the following association. $2(2)+1(3)+1(1)$. Note the association of rod bivalents of different sizes. Fig. 71. *C. arietinum*. Metaphase I, showing a ring of four chromosomes. Fig. 72. *C. arietinum*. Metaphase I, showing 6_n and 1_{IV} . Fig. 73. *C. arietinum*. Metaphase I, one bivalent is lying off the plate. Fig. 74. *C. arietinum*. Metaphase I, univalents are lying away from the plate. Fig. 75. *C. arietinum*. Side view of Metaphase I. One bivalent lying across the plate. Fig. 76. *C. arietinum*. Anaphase I showing several univalents lagging. Fig. 77. *C. arietinum*. Telophase I. Univalent lagging and divided. Fig. 78. *C. arietinum*. Zygotene stage showing cytomixis. Fig. 79. *C. arietinum*. Prometaphase II, showing cytomixis. Fig. 80. *C. arietinum*. Metaphase II showing cytomixis. Fig. 81. *C. arietinum*. Sixteen prochromosomes in a root-tip cell and a nucleolus. Note some of these are in pairs. Fig. 82. *C. arietinum*. Polar view of early telophase in somatic division. Prochromosomes forming. (Optical section). Fig. 83. *C. arietinum*. Late telophase. Prochromosomes and reticulum fully formed. A single nucleolus seen. Fig. 84. *C. arietinum*. Optical view of the premeiotic cell, showing some of the prochromosomes. Note the radiating processes starting from each prochromosome. Fig. 85. *C. arietinum*. Optical view of the premeiotic pollen mother-cell showing the prochromosomes. Note the diverging processes in each prochromosome on either side of the spindle attachment region. Note numerous pale staining masses of chromatin. The nucleolus is shown in outline (optical section). Fig. 86. *C. arietinum*. Optical view of the premeiotic cell. Prochromosomes are practically lost and the chromatin masses have become more chromatic, and some of these are in lines. Fig. 87. *C. arietinum*. A later stage than Fig. 86. Leptotene threads are seen forming here and there (optical section).

layer throughout meiosis. In certain abnormal cases the tapetal cells were very much elongated and invaded the loculus of the anther (Text-fig. 65). The pollen grains were shrunken and sterile.

Pollen-grain divisions. The first division of the pollen grain takes place long before the opening of the flower. Text-figs. 66–9 show stages of division. Prochromosomes are present.

VII. GENERAL CONSIDERATIONS

(a) Secondary association.

In secondary pairing the bivalents are attracted mutually but connexions are never formed between them. The attraction at second metaphase is more marked in certain plants, as *Solanum* (Muntzing, 1933), where the chromosomes are relieved of all the tensions existing at the first division. Secondary association has no counterpart at diakinesis. The mechanism which brings it about has been described by Lawrence (1931a) in *Dahlia* and Catcheside (1934) in *Brassica*. In the case of multivalents, on the other hand, the association is seen at diakinesis, where the members have organic connexion which at anaphase may cause disturbance.

In the present case, at diakinesis, regularly eight bivalents are formed (Text-fig. 40a). Occasionally a trivalent and a univalent were seen (Text-fig. 40b). The side view of the spindle showed in some cases association between a long and a short rod bivalent (Text-fig. 70), indicating an affinity between non-homologous chromosomes which have some segments in common. A similar type of association is seen in *Brassica* (Richharia, 1937). In polar view pairing of bivalents of different sizes was seen, (Text-fig. 47). Both in polar view and side view association of three bivalents was seen (Text-fig. 70). The maximum association met with was 1(3)+2(2)+1(1) (Pl. VII, Fig. 6). The most common arrangement is 3(2)+2(1) (Table I and Text-fig. 43 and Pl. VII, Fig. 5). In side view one instance of a ring of four chromosomes (Text-fig. 71), and a quadrivalent in which the members were associated terminally to give an X-shaped configuration (Text-fig. 72), were also seen. The observations indicate that structural changes have played a part during the evolution of this species, in addition to polyplody. The diploid number (16) may therefore be represented as follows:

$$A_1A_1A_2A_2; B_1B_1B_2B_2; C_1C_1C_2(C_2d_2); D_1D_1D_2(D_2c_2)$$

assuming that segmental interchange has taken place between chromosomes C₂ and D₂. An association of C₂, (C₂d₂), D₂, (D₂c₂) leads to a ring of four chromosomes, or an X-shaped quadrivalent. An association of four bivalents, i.e. C₁C₁; C₂(C₂d₂); D₁D₁; D₂(D₂c₂), is possible. In the present investigation, associations of four bivalents were not seen, but groups of three bivalents

were met with. Thus the maximum association of $1(3)+2(2)+1(1)$ may be represented as follows:

A_1A_1	A_2A_2	B_1B_1	B_2B_2	C_1C_1	$C_2(C_2d_2)$	$D_2(D_2c_2)$	D_1D_1
2	2				3		1

Thus the basic number of *C. arietinum* may be represented as four.

TABLE I

Types of Secondary Association as Meiosis

No. of secondary associations.	No. of bivalents in association.			No. of cases.	Total.
	1.	2.	3.		
0	8	—	—	8	8
1	6	1	—	9	
1	5	—	1	3	12
2	4	2	—	13	
2	3	1	1	3	16
3	2	3	—	24	
3	1	2	1	1	25
4	—	—	—	—	—
				Grand Total	61

The pairing completely disappears in the late anaphase and telophase. At Metaphase II the chromosomes are much longer and are paired like somatic chromosomes in root-tips (Text-fig. 60). Sansome and Philp (1932) take the view that secondary pairing and somatic pairing are analogous. According to Lawrence (1931a), Muntzing (1933), and Nandi (1936), secondary association is more marked in the second division in the materials examined by them.

(b) Irregularities in meiosis.

Failure of congression of the chromosomes, which has been attributed to an upset in timing balance, has been reported in *Avena* hybrids (Philp, 1933), male-sterile *Lathyrus* (Upcott, 1937), *Podophyllum* (Darlington, 1936), &c. Congression may be hindered by the repulsions of the other chromosomes on a crowded plate, and in this case one bivalent may form an accessory plate half-way between a primary plate and a pole (Darlington, 1936). According to Koller and Darlington (1934), the sex chromosomes are particularly liable to behave in this way. Bivalents that do not congress also do not orientate. Non-orientation may also be caused by interlocking and multiple association.

In the present material a particular bivalent is seen to pair only at the ends. In Text-fig. 73 a side view of metaphase is shown where one bivalent is outside the crowded metaphase plate. In certain cases two univalesst seen side by side outside the plate (Text-fig. 74). The pairing place without chiasma formation. The affected bivalent is a rod-bi⁴ also seen by Darlington (1937) in *Tradescantia*. A ring bivalen⁴

fail to congress, as seen by Philp (1933) in *Avena* hybrids and by Philp and Huskins (1931) in *Matthiola incana*. In one case a rod-bivalent was seen right across the plate (Text-fig. 75).

Univalents may also be formed. They arise in this material from two causes: (1) failure of pairing of short chromosomes, as mentioned above; (2) occasional formation of a trivalent. In one case a large number of univalents were seen lagging at anaphase I (Text-fig. 76). The behaviour of univalents arising by any of these methods is the same as is that of bivalents which fail to congress on the plate. The univalents invariably approach the plate much later than the bivalents. If they are very near to the plate they arrange themselves on it and split and the two halves may pass to the poles (Text-fig. 54). Sometimes they may remain outside (Text-fig. 77). If the univalents are far from the equator they may be included with the daughter chromosomes at one of the poles. These divide in the second division, and they may lag in that division. The lagging univalents do not give rise to micronuclei but are lost in the cytoplasm.

Cytomixis. The phenomenon of cytomixis was first described by Gates (1911b) in *Oenothera gigas*, where he showed the transference of chromatin between two contiguous cells taking place through gaps in the cell walls by means of protoplasmic connexions. This process has since been reported in many plants. Kattermann (1933) refers to these works and shows various stages of cytomixis in the pollen mother-cells of *Triticum* × *Secale* hybrids. In the present case cytomixis was seen both in the first and second divisions. Text-fig. 78 shows the cytomixis in prophase at zygote. In Text-fig. 79 two pollen mother-cells are shown in which a part of the chromatin has passed from one into the other. The extruded chromatin probably passed through the wall in the thread condition and has since condensed into chromosomes which retain their individuality. In Text-fig. 80 is shown a similar transference in the second division. Similar behaviour in various stages of meiosis has been reported by Gates and Latter (1927) in *Lathraea*, where in addition to prophase it also occurs in interkinesis. Church (1929) reports it in grasses. Cases have been reported where by cytomixis an increase in chromosome number has been brought about. Nandi (1937), in rice, found one case in which the whole of the nuclear contents had passed from one mother-cell into the other, forming a binucleate pollen mother-cell at diakinesis. Such cases have also been reported by Kihara and Lilienfeld (1931) in certain hybrids between *Triticum* × *Aegilops*.

(c) Prochromosomes.

These bodies have attracted the attention of various workers and various hypotheses have been given to them. The term 'prochromosome' suggested by Balfour (1905) has the widest usage. Smith (1934) and Manton (1935) have reviewed the various views held by previous workers. The former has approached the problem from three points: (1) As regards the nature of the pro-

chromosomes themselves, he agrees with the views of Heitz (1929), Gregoire (1932), and Doutreligne (1933) that a prochromosome represents a portion of the chromosome on either side of and adjacent to the spindle attachment region. (2) He concludes that the number of prochromosomes in a nucleus may vary and the region on either side of the spindle attachment region may become achromatic. Thus all nuclei do not exhibit the full diploid number of prochromosomes. There is no pairing or fusion of prochromosomes in either somatic or premeiotic nuclei. (3) Threads are regularly present in resting and meristematic nuclei, and at least some of them represent chromonemata. On the other hand, Gregoire (1932) and Doutreligne (1933) hold that chromonemata are not present in plants with prochromosomes. Smith (1934) concludes that the structure of the chromosomes and the chromosome cycle in plants with prochromosomes are fundamentally the same, in both the somatic and meiotic divisions, as those without prochromosomes and with larger chromosomes. The formation of leptotene strands is similar to that of the single strands in the prophases of somatic divisions. These strands may show prochromosomes and primary constrictions until they pair side by side during synapsis.

Manton (1935) classifies small nuclei showing prochromosomes as 'vesicular' while large nuclei of ordinary type are 'solid'. By examination of the root-tips of diploid and tetraploid species of *Biscutella laevigata* and also the nuclei of the tetraploid chimeral areas, she showed a numerical correspondence between the chromosome numbers and the number of prochromosomes. In the vesicular type extensive movements are involved in the conversion of a flat anaphase plate into the spherical resting nucleus.

In the present material prochromosomes are seen in the resting nuclei of root-tip cells, in the premeiotic pollen mother-cells, and tapetal cells. In root-tip material fixed with Navashin's solution the number of prochromosomes agreed with the chromosome number (Text-fig. 81). But variations were often noticed. This may be due to the fact that one or two small prochromosomes were too faint. As in the metaphase, size differences were also noticed. In the tetraploid chimeral areas a rough approximation was obtained. In premeiotic and in tapetal cells the prochromosomes showed a number of radiating processes (Text-figs. 84, 85). As already stated, in the root-tip cells at anaphase the two arms of the chromosomes on either side of the spindle attachment region gradually contract (Text-fig. 8) till at late anaphase in polar views the two limbs are so markedly reduced that they appear as a pair of spherical bodies one on either side of the spindle attachment region (Text-fig. 9). The characteristic flat anaphase plates are maintained until late anaphase, as seen by Manton (1935) in *Biscutella*. In telophase deep-staining masses are seen, and connecting these there are broad strands which take up a lighter stain (Text-fig. 82). The chromatic bodies gradually increase in size and the lightly staining connecting areas appear as thread-like processes (Text-fig. 10). At still later stages the bodies become more distinct and the

strands show a reticulate appearance (Text-fig. 83). Thus in the resting nucleus the chromatic bodies and the faintly staining reticulum and a single large nucleolus are seen. The origin of the nucleolus in the early telophase could not be identified with satellites. At later stages two or three nucleoli are seen, which gradually fuse to form a single body.

In the premeiotic nuclei, with the onset of prophase the nucleus shows a large number of chromatic bodies in addition to the prochromosomes (Text-fig. 85). It is difficult to show all the details in the drawings as the bodies lie in several planes. At later stages the reticulum is practically lost and chromosomes are seen forming here and there by the gradual accumulation of chromatin on either side of the spindle attachment region (Text-figs. 86, 87). Later, the threads are fully formed. They are long and thin and curve in all directions.

Thus the present material shows that prochromosomes are portions of chromosomes continuing from telophase and persisting in the resting nucleus as chromatic masses on either side of the spindle attachment region, as observed by Smith (1934). The plate-like arrangement of the chromosomes which is seen in late anaphase is disturbed in telophase by their migration to the periphery, thus enabling the fusion of the nucleoli at later stages. The chromosome individuality is never lost, and excepting for some of the differences as detailed by Manton (1935) the cycle of chromosomes is alike in nuclei with prochromosomes and those without these bodies. Recently Raghavan (1937) has followed the cycle of prochromosomes in *Polanisia trachysperma* and *Gynandropsis pentaphylla* and finds in his material that the behaviour of the nuclei containing prochromosomes is essentially like those plants without prochromosomes.

VIII. DISCUSSION

(a) Relationship of the nucleolus and chromosomes.

The extensive literature concerning the nucleolus has been well summarized by previous workers. Nandi (1937) in rice, and Bhatia (1938) in wheat, have discussed at length and summarized the various views held from time to time regarding this question. Gates (1937) has reviewed the stages of this discovery of the relation between satellites and nucleolus.

In the first place, Latter (1926), in *Lathyrus*, found the spireme in contact with the nucleolar body. A similar observation was made in *Oenothera* (Sheffield, 1927) and other plants. Navashin (1912), in *Galtonia*, discovered the satellites attached to the nucleolus. He thought that these bodies later became attached to the chromosomes by fine processes sent out by the latter. Further observations, however, show that the satellite was part of the chromosome. Wenrich (1916) in certain grasshoppers, Sorokine (1924, 1929) in *Ranunculus*, Heitz (1931b) in *Vicia*, Derman (1933) in *Callisia*, Smith (1933) in *Galtonia*, Nandi (1937) in rice, Bhatia (1938) in wheat, all showed the

attachment of certain chromosomes to the nucleolus by satellites, the number of attachments being equal to the number of satellites.

Heitz (1931) found that the nucleolus originates from the achromatic thread of the satellite chromosomes, the number of nucleoli corresponding with the number of satellites and secondary constrictions present. McClintock (1934) in *Zea mays* established that the nucleolus arises from the nucleolar organizing body, situated immediately adjacent to an achromatic region delimiting a satellite in one chromosome (chromosome VI) of the haploid complement, the material for the formation of the nucleolus being probably contributed by all the chromosomes. Nandi (1937), in rice, and Bhatia (1938), in wheat, report that the organizing body is terminal.

The number of nucleoli organized at telophase and the number of sets in the chromosome complement has been reported to agree in many plants, i.e. in the haploid there is one satellite and one nucleolus is formed at the telophase, in the diploid the number of nucleoli formed is two, in triploid three, and so on. De Mol (1928) in *Hyacinthus*, McClintock (1934) in *Zea*, Bhatia (1938) in wheat, and others have all shown such a relationship.

Some have attempted to find out whether the substances in the nucleolus and in the chromosomes are identical and whether there is any direct flow of the nucleolar material into the chromosomes. According to the earlier view of the continuous spireme theory, it was usually held that the nucleolus functioned as repository of chromatin material which was stored in telophase and resting stage and used in the prophase. Fikry (1930) and Derman (1933) pointed out the difficulty of such a direct transference when the spireme was found not to be continuous. Microchemical tests carried out by Zacharias (1882), Yamaha and Sinoto (1925), and Zirkle (1928, 1931) show that nucleolar substance is not composed chemically of 'nucleic acid', 'chromatin', or 'karyotin'. Two substances appear to be present in the chromosome, one of which is of nucleolar origin. Zirkle (1928, 1931), Marshak (1931), Derman (1933), McClintock (1934), and Nandi (1937) are of the opinion that the nucleolar substance contributes to the matrix of the chromosomes. Thus the nucleolus is a compound body consisting of substances from all the chromosomes. Possibly the transference of material from the nucleolus takes place by its solution in prophase followed by its reconstitution on the matrix of the chromosomes. At telophase again the globules of material derived from the matrix of the chromosomes do not appear to be carried bodily to the young nucleolus at the nucleolar locus of one pair of chromosomes, but rather to be dissolved or chemically changed and reconstituted in the nucleus under the influence of the nucleolar body.

Some have attached significance to the size of the vacuoles in the nucleolus and the increase in chromaticity of the chromosomes during prophase. It is generally seen that the vacuoles at the resting stage are small, but in the late prophase they are considerably larger. The observations of Wager (1904) in *Phaseolus*, Latter (1926) in *Lathyrus*, De Mol (1926) in *Hyacinthus*,

Sheffield (1927) in *Oenothera*, Derman (1933) in *Callisia*, show such behaviour.

All the six points mentioned above show that there is an intimate relationship between the chromosomes and the nucleolus. In the present study of *Cicer arietinum* the nucleolus is a prominent body and is always single in the resting condition. The vacuolation of the nucleolus increases with the progress of prophase in both the somatic and meiotic divisions. Occasionally crystalline bodies, as seen by Latter (1936) in *Lathyrus*, Sheffield (1927) in *Oenothera*, and others, are visible. In the somatic metaphase four chromosomes bear satellites. In *C. soongaricum* four satellites are present, two big and two small (Text-fig. 7). In *C. arietinum* in the resting nucleus four prochromosomes and four chromosomes in the somatic prophase are seen attached to the nucleolus (Text-figs. 3, 21). At meiosis the situation is as follows. In leptotene four threads are seen attached to the nucleolus (Text-fig. 20). At zygotene and pachytene if the pairing is complete then two bivalents are attached to the nucleolus.

The intimate relationship between the chromosomes and the nucleolus can be shown in another way. In the early zygotene and pachytene stages the nucleoli often develop buds, the significance of which is not yet well understood. In such cases a chromosome is always seen to pass between the main nucleolus and the bud at the point of junction. Such behaviour has been reported in rice by Selim (1930) and Nandi (1937) and in other plants.

Cases of nuclei having satellites of different size, as in these species of *Cicer*, are known. S. Navashin (1912) found in *Galtonia candicans* two different types of satellites, and Taylor (1926) in *Fritillaria imperialis* observed two pairs of metaphase chromosomes having distal satellites of different sizes. Fernandes (1935), on the other hand, denies the existence of symmetrical and asymmetrical races in *Narcissus*, but thinks that the size of the satellite is variable in the cells of the same individual. He divides satellites into two types—heterochromatin satellites and euchromatin satellites. Mather (1932) in *Crocus sativus* says that genotypic changes are responsible for the length of the satellite thread.

(b) *Cicer arietinum* considered as a polyploid.

In the absence of knowledge of the chromosome numbers and the behaviour of meiosis of the other species of this genus, the evidence to support the view that the cultivated *Cicer arietinum* is a polyploid with a basic number lower than eight can be arrived at only by circuitous methods. That duplication of chromosomes has probably taken place in the evolution of this species may be inferred from the following evidence: (1) secondary association; (2) occasional formation of trivalents and quadrivalents; (3) the constant attachment of four chromosomes to the nucleolus and the presence of four satellites in the somatic metaphase plate in *C. soongaricum*; (4) duplicate factors in the genetical behaviour.

Secondary association is believed to be a criterion of homology of the associated bivalents in a number of plants, and has been used to determine the basic number of the species, in haploids, diploids, or polyploids. By this method it has been shown that some of the reported haploids, as rice (Nandi, 1936), have not the real basic number. Similarly, in some of the apparent diploids, reduplications of single chromosomes have taken place resulting in fertile species. These are thus secondarily balanced diploids, as in *Tricyrtis*, $n = 13$ and *Dicentra*, $n = 8$ (Matsuura, 1935b), which are derived from basic sets of 12 and 7 respectively. Similarly in *Acer*, $n = 13$ (Meurman, 1933), the true basic number is 12. Some of the apparently diploid species are in reality polyploids and some have undergone structural changes. They are thus secondarily balanced polyploids. In rice (Nandi, 1936) the $2n$ number 24 is derived from a basic number 5 and thus the apparent diploid is really a secondarily balanced tetraploid. Similar cases are seen in *Gossypium* (Davie, 1933), where $2n = 26$ is probably derived from 7; *Brassica*, $b = 6$ (Catchesside, 1934, Alam, 1936); *Solanum tuberosum* (Muntzing, 1933), where $2n = 24$ is derived from $b = 6$. In the section *Pomoideae* of Rosaceae it has been shown that the haploid number 17 is derived from a basic number 7 (Moffett, 1931). Lawrence (1931) has discussed at length the relation between secondary association and allopolyploidy. According to him, secondary association may under exceptional circumstances be met with also in autopolyploids.

In the present study it has already been pointed out that the maximum association is $1(3)+2(2)+1(1)$. The most frequent association is $3(2)$ and $2(1)$. No case with $4(2)$ was obtained. The possibility of segmental interchange having played a part in the evolution of *C. arietinum*, besides polyploidy, has already been pointed out. The association of three bivalents and the formation of a ring of four chromosomes have been ascribed to this structural change. The regular formation of rings met with in the ring-forming types, as *Datura* (Belling and Blakeslee, 1924, 1926), *Pisum* (Hakansson, 1931, and others), &c., is met with in this case very rarely. Similarly, Afify (1933), in *Aconitum*, found regular formation of bivalents, rings forming very rarely. According to him this behaviour is due to the fact that only small segments of the chromosomes have interchanged. Accepting such an explanation in the present case, the small portions of the chromosomes that have interchanged can make an association of more than two bivalents possible. Associations of more than two bivalents have been reported in *Brassica* (Richharia, 1937) and other plants. Matsuda (1934), in giant Petunia, explains the multivalent associations and abnormal configurations by the segmental interchange hypothesis. In the case of *Solanum tuberosum* Muntzing (1933) found a maximum association of $5(2)$ and $2(1)$ and says that probably 6 is the basic number. In the present case, if we assume 4 as the basic number, then *C. arietinum* and *C. pinnatifidum* are really secondary tetraploids.

If we represent the original constitution of the haploid set by A, then by

gene mutation or structural changes another type having a different constitution, say A₁, may arise from the original. A cross between the two might be partially sterile (*Nicotiana Bigelovii* \times *N. suaveolens*, Goodspeed and Clausen, 1927) where there is a low frequency of pairing. If, in the cross A \times A₁, doubling of the chromosomes takes place either somatically, as in *Primula Kewensis* (Newton and Pellew, 1929) and *Nicotiana digluta* (Claussen, 1928), or gametically as in *Raphano-Brassica* cross (Karpechenko, 1927, 1929), &c., it would be fertile, vigorous, and constant through autosyndesis. We may assume that the species of *Cicer* with 16 chromosome numbers have probably arisen in this way.

In the case of *C. soongaricum*, which has 14 chromosomes, the 4 satellites, 2 big and 2 small, indicate allopolyploidy. The 14 chromosomes may have been derived from 16 through meiotic irregularities, or independently from 4 chromosomes by secondary allopolyploidy. A similar case with 7- and 8-series has been reported in *Ranunculus* (Larter, 1932).

The genetical studies of *C. arietinum* have shown a complex behaviour in a number of characters (Shaw and Abdul Rahman-khan, 1931). Thus the evidence that *C. arietinum* is an allotetraploid is strongly indicated.

IX. SUMMARY

1. An examination of the root-tips of 30 varieties of *C. arietinum* showed 16 chromosomes in each case. No morphological differences were noticed. The root-tips of two varieties of *C. soongaricum* showed 14 chromosomes, which were found to be uniformly much bigger than in *C. arietinum*. This indicates the possibility of two polyploid series within the genus *Cicer*, as in *Ranunculus*.

2. The resting nucleus of *C. arietinum* showed conspicuous prochromosomes which were not seen in *soongaricum*. In *C. arietinum* 4 prochromosomes are attached to the nucleolus in the resting nucleus and 4 chromosomes to the nucleolus in somatic prophase. In the somatic metaphase 4 satellites are seen. In *C. soongaricum* 4 satellites are present, 2 small and 2 large. This indicates allopolyploidy in these species.

3. Irregularities in the somatic division, as somatic doubling, lagging chromosomes, and persistence of the nucleolus in the metaphase were observed. Somatic pairing occurred in both species.

4. The prochromosomes are the persistent portions of chromosomes on either side of the spindle attachment region. The cycle of mitosis and meiosis in the nuclei showing prochromosomes is identical with the nuclei without prochromosomes. The special features of meiosis in *C. arietinum* are (1) an extreme diffuse stage in diplotene, (2) secondary association in Metaphase I.

5. An analysis of secondary association in Metaphase I shows a maximum association of 1(3)+2(2)+1(1). This indicates that 4 is the basic number. Segmental interchange has also probably played a part in the evolution of

this species. *C. arietinum* is an allotetraploid with structural changes. The attachment of 4 chromosomes to the nucleolus in the leptotene and 2 bivalents in zygotene, pachytene, and diakinesis is another indication that duplication of chromosomes has taken place in *C. arietinum*.

6. Irregularities in meiosis, as failure of pairing, non-congregation and non-orientation of the bivalents on the metaphase plate, premeiotic non-disjunction and cytomixis, were seen.

CV

X. ACKNOWLEDGEMENTS

I wish to acknowledge my deep indebtedness to Professor R. R. Gates, and express my grateful thanks for his kindly interest and thoughtful criticism throughout the progress of this investigation. I am indebted to Mr. C. S. Semmens, Technician, Botany Department, for several kinds of help rendered during the course of this work.

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EXPLANATION OF PLATE VII

Illustrating Mr. N. K. Iyengar's paper on 'Cytological Investigations on the Genus Cicer'.

(All figures are from photomicrographs)

Fig. 1. Seeds of some of the varieties of *C. arietinum* examined.

		Seed Colour.
1.	Kabuli variety. (London market sample)	White.
2.	" Strain no 2. Coimbatore.	"
3.	" From Germany.	"
4.	Indian variety. Lyallpur no. 6.	Yellowish Brown.
5.	Var. <i>rotundum</i> . (Germany).	Reddish brown.
6.	Var. <i>nigrum</i> . "	Black.

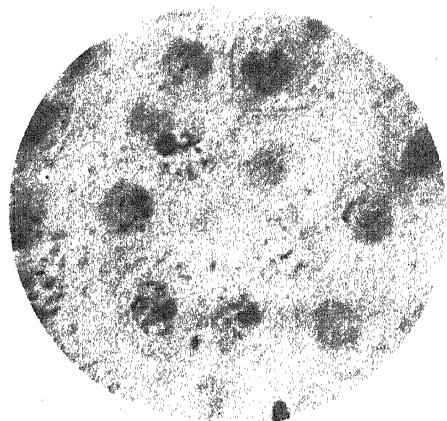
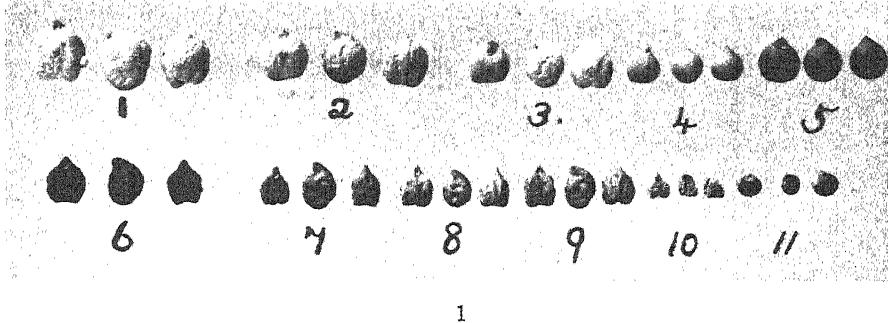
- | | | | |
|-----|--------------------------|-----------------------------|---------------|
| 7. | Indian variety. | (Lyallpur no. 17). | Bluish brown. |
| 8. | " | (Lyallpur no. 7). | Light brown. |
| 9. | " | (Coimbatore 468). | Dark brown. |
| 10. | <i>C. pinnatifidum</i> . | —Note the spiny seed coat. | |
| 11. | <i>C. Montbretii</i> . | —Note the smooth seed coat. | |

Figs. 2 and 3. Various stages of diffusion at diplotene. $\times 1,600$.

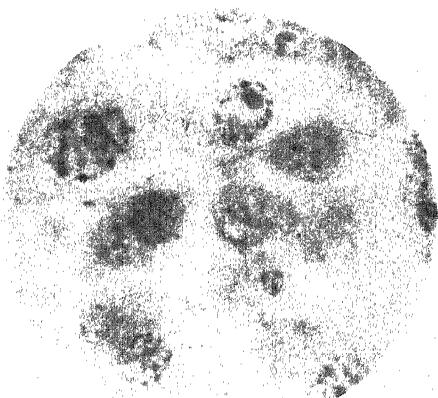
Fig. 4. Extreme diffusion. $\times 2,000$.

Fig. 5. Secondary association at metaphase I. in *C. arietinum*. 3(2)+2(1). $\times 2,000$.

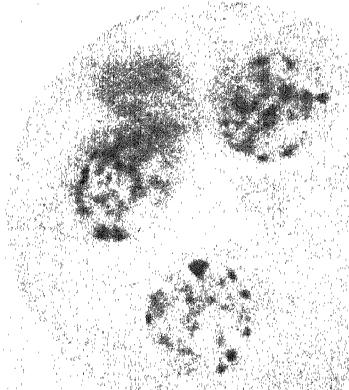
Fig. 6. Secondary association at metaphase I. in *C. arietinum*. 1(3)+2(2)+1(1). $\times 2,000$.



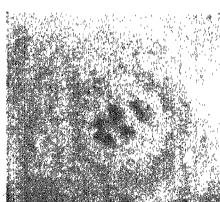
2



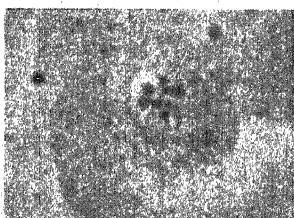
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4



5



6

Structural Abnormalities in Cotton Leaves following Exposure of the Seed to X-radiation

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I. INTRODUCTION

THE study of the effect of X-rays upon green plants has been engaging the attention of biologists and radiotherapists since 1895. Investigations have so far led to the view that these rays even in minute doses (Singh and Choudhri, 1935) are markedly effective in initiating various modifications in plants. A further perusal of the literature indicates that all plants are not similarly sensitive to the action of these rays (Johnson, 1936). Observations are also on record to show that the degree of response even differs in parts of the same plant (Duggar, 1936). Roots, for instance, are observed to be more susceptible to the forcing action of these rays than are the stems, the lateral being found to be more susceptible than the main roots. The damage characteristic of X-ray treatment of plants is, however, never more conspicuously shown than by the leaves. X-rays are so powerful that the anomalies following exposure to them are not surprising, for under the direct influence of such rays variations (e.g. Goodspeed, 1929, and Wolcott, 1936) are well known to occur. The present work was, therefore, directed to determine the abnormalities in leaves of plants following exposure of the seed to X-radiation. Furthermore, to gain an insight into the sensitivity of the seeds under different conditions, viz. dry, soaked, and germinated, the extent of this investigation was trebled.

II. PROCEDURE

In order to focus the details of this investigation, the observations were preferentially confined to one aspect of one crop. The crop chosen was cotton, reliable seeds of var. C. 520 being procured. This was due to the co-operation of Dr. B. L. Sethi, Economic Botanist to the Government of the United Provinces, to whom the authors are grateful.

Before treatment the seeds were divided into three lots. One was maintained dry, while the other two were soaked in distilled water for periods of 24 and 48 hours respectively. In the second lot, after 24 hours' soaking only a few seeds sprouted which were removed, so providing an entirely homogeneous, soaked, and ungerminated lot for treatment. Of the seeds soaked for 48 hours, only a few failed to germinate which were also removed, so that the third lot consisted of soaked, germinated seeds.

The three lots thus obtained were exposed to X-rays, but from each a portion was retained to act as a control. The 'set up' of the tube used in this work is as follows: voltage = 54 K.V.; tube current = 5 ma.; distance = 15 cm.; anticathode = tungsten; duration of irradiation = 5, 15, and 30 minutes respectively.

With a view to studying the effect of varying doses of X-radiation, the time of exposure was varied from 5 to 30 minutes, while the rest of the conditions of the 'set up' were maintained strictly identical.

Seeds after treatment were planted in pots containing a mixture of soil and manure. The effects of irradiation were best seen during the first month after germination. The affected leaves from the rayed plants and the corresponding ones from the controls were, therefore, detached one month after germination and fixed with formalin-acetic-alcohol (Rawlins, 1933). Microtome sections (10μ) were made of the leaf blades (passing through the midrib) and of the petioles.

III. OBSERVATIONS

Johnson (1936) classed in relation to X-rays seventy species of plants in three groups, as (i) resistant, (ii) slightly susceptible, and (iii) highly susceptible. If that scheme of classification is to be followed, the cotton plant falls in the last-named group, for the modifications characteristic of that group were well apparent even externally, and more so on the histological examination of the leaves of the radiated plants. The degree of response of leaves differed greatly according to the strength of the dose and to the state of the material during treatment. Milder doses did not cause such appreciable variations as the heavier ones. Similarly, dry seeds were less susceptible than the soaked or germinated ones.

Morphological.

The leaf stalks of plants from seeds treated dry, though thicker than the control, were obviously little affected, but those where irradiation was given to

the soaked or germinated seeds presented a peculiarly grooved aspect. This condition was more pronounced when the doses were comparatively heavy, viz. those of 15–30 minutes duration. Such petioles were shorter and had a smaller circumference than the corresponding controls.

Developmental irregularities were more common and more apparent in the leaf blades of the plants grown from X-rayed seeds. Plants from seeds irradiated dry were not much affected in this respect. Heavy doses of the soaked seeds, on the contrary, caused many deformities in the leaf lamina. The blades of the first few leaves were asymmetrical, often bore tumour-like structures, and had a distorted margin. Anomalies in the leaf shape of irradiated plants were also reported by Horlacher and Killough (1931).

Cotton leaves are usually lobed, but heavily-dosed plants often produced entirely unlobed leaves. The lamina of such leaves had a number of puckering and often presented a variegated appearance. The presence of blue-green spots intermingled with light green further indicated that such treatments had caused chlorophyll disturbances to a not inconsiderable extent.

Petiole.

Before arriving at any conclusion regarding the histological effects of radiation, it is necessary to examine and deal with the microscopic details of the leaves belonging to normal plants. Unlike many plants, those of normal cotton, more specially of the variety investigated and under the conditions in which these plants were grown, bore leaves with more or less cylindrical petioles without a groove. At places, however, their margin showed a slight waviness, presumably due to fixing. The essential parts of T.S. of such leaf-stalks may briefly be noted as an epidermis, a single-layered hypodermis, several-celled parenchymatous cortex, a ring of four collateral bundles, medullary rays, and a distinct pith.

A number of variations were apparent on sectioning the leaf-stalks of plants raised from irradiated dry seeds, most common of which were absence of a hypodermal layer, greater development of ground tissue resulting from an increase in cell size and cell number, and reduction in the number of bundles from 4 to 3, which were now arranged along a curved band instead of in a ring. The two upper bundles were always smaller than the middle one which was apparently double the size of each of these, indicating the possibility of two bundles having merged to form one. Notwithstanding such modifications the petioles were round like the controls.

The petioles resulting from treatment of the soaked seeds were readily distinguished from both the untreated and also from those from plants from treated, dry seeds. Such stalks evidently possessed a dorsiventral structure with their upper surface either flattened or slightly grooved. This, in fact, is the normal form of many leaf-stalks though not of cotton. The ground tissue was much less developed than in the control, and although most of the cells were parenchymatous, bands or ridges of sclerenchyma

were not infrequently developed beneath the epidermis. As with the treatment of dry seeds the three dissimilar bundles were again arranged in a curved band. This treatment was further characterized by a greater development of the xylem element. The middle bundle had, as an average, 9 rows of pitted and spiral vessels with about 5 vessels in each row. In the control, on the contrary, the rows were only 5–6, with 3–4 vessels in each of them. On subjecting the soaked seeds to a longer treatment of 30 minutes the tissue system of the leaf-stalks became greatly deranged and also ruptured at several places. The disintegration was more clearly exhibited in the longitudinal sections of such petioles.

The petioles of leaves of plants grown from irradiated germinated seeds though more or less similar in general appearance (external as well as internal) to those raised from soaked ungerminated seeds, still had a peculiar vascular system. Instead of four bundles in the control and three in the other two irradiated sets, there was but one continuous crescent-shaped bundle with highly developed wood. This rather extraordinary arrangement suggested that such a condition resulted from the merging of several bundles. Doubtless the seeds in the germinated state surpassed the two previous sets in their sensitiveness to irradiation.

Lamina.

The modifications of the lamina following irradiation differ strikingly from those of the petiole. Unlike the petioles the leaf blades from irradiated sets of plants were characterized by greater development and increasing disintegration of the mesophyll. Material from the treated soaked and germinated seeds was most affected and though slightly less developed these were more disintegrated than the others. With the treatment of dry seeds, on the contrary, the blades were more developed but showed less injury, comparatively. These effects increased with larger doses.

The palisade parenchyma in a normal leaf consists of compact columnar cells set at right angles to the upper epidermis. Most of these cells were misplaced as a result of irradiation and occupied all sorts of positions between the normal, at right angles to the epidermis, and one parallel with the epidermis. Many of these cells overlapped each other so that the tissue lost its homogeneity and many large spaces, uncommon in normal leaves, were to be observed. The crooked appearance and the elongation of the palisade cells suggested that they had undergone some sort of compression as a sequence to irradiation. The extreme cases arise where a long treatment is given to soaked and germinated seeds, resulting in the complete disappearance of palisade cells, presumably due to their splitting up to form smaller stellate cells resembling those of spongy parenchyma. Noguchi (1935) in his work on sunflower also pointed out similar changes in the structure of the leaves. Such a condition is normally found in some monocotyledonous leaves (e.g. those of lilies) which stand more or less erect.

Of the many important structural differences in the leaves brought about by seed irradiation, those in the number and development of fibro-vascular bundles were most striking. In petioles the number of bundles was reduced from three to one, under different types of seed irradiation. In leaf blades, on the contrary, the results were the opposite, the bundles increasing from one to three under the same circumstances. It may be noted that on irradiation the reduction in the number of bundles in petioles showed a closer and closer placing as the treatments changed from that of dry seeds to soaked, and soaked and germinated seeds. When the bundles were far apart in the petiole, and more so in the blades (e.g. in cases where dry seeds were treated), the microtome sections of blades included but one of the three bundles actually present. With the other two treatments, the bundles came in closer contact allowing the inclusion of two and three bundles respectively. Treatment of soaked and germinated seeds which caused the bundles to combine in the petiole produced three of them in the lamina. This is to be explained as a result of splitting, though the bundles retain their positions close to each other.

In normal leaves the spongy parenchyma above and below the vascular bundles is well developed, and often produces a protuberance on each side of the leaf. On irradiation, the development of the spongy parenchyma in that position was greatly affected, such that the lower surface of the leaf, though consisting of larger cells, hardly showed any protrusion below the bundle. The upper surface, though not much affected under some treatments, was concave in extreme cases. On the whole, the structure in control leaves was more or less symmetrical along the midrib, but much of the symmetry was lost in leaves of plants from irradiated seeds.

IV. DISCUSSION

The powerful action of X-rays upon cotton is well shown by the transmission of the many structural variations from seed to plant. Most of the external changes could easily be explained on microscopical examination of the tissues. Overlapping and unequal development of the chlorophyll-bearing tissue seem to cause puckering. Changes in this tissue, due to over-stretching in case of mild doses and to killing of some cells in heavy doses, is held responsible for the disintegration observed.

It may also be noted that on X-raying the seeds the changes initiated in the lamina and petiole were never the same. As stated earlier sensitiveness to X-rays differs not only from plant to plant but from part to part of the same plant. The lamina was more sensitive than the petiole.

For obvious reasons the injurious effects of a mechanical nature were more evident in the lamina. No two tissues showed the same response to irradiation, palisade parenchyma exhibiting the greatest injury. In general, the xylem was much more developed in the treated plants. Miege and Coupe (1914) likewise found the vascular tissue of rayed *Raphanus* and *Lepidium* better than that of the controls.

Sensitiveness was found to vary with the amount of moisture present in the tissue during irradiation, i.e. probably with the metabolic rate. Thus the effect on plants from dry seeds was less than that from soaked seeds, still less than that from the germinated seeds.

V. SUMMARY

Dry, soaked, and germinated seeds were exposed to X-rays for 5, 15 and 30 minutes and the plants then raised in pot culture under similar conditions.

Irradiation of seeds for 5 minutes caused some modification in the leaves and this increased with increasing exposure.

The effect on leaves of plants raised from dry irradiated seeds was less than with soaked seeds and the germinated seeds showed a still greater effect.

Petioles after dry-seed treatment were thicker, while they became grooved in the cases of irradiated, soaked, and germinated seeds.

No external change was noticed in the leaf lamina following the treatment of dry seeds. The treatment of soaked and germinated seeds gave an asymmetrical lamina which was variegated and showed puckerings and tumour-like structures.

Petioles of normal leaves were cylindrical, but with heavy irradiation they became dorsiventral. The conducting tract also changed from several bundles in a ring to a single crescent-shaped mass, and this was associated with greater development of wood.

The vascular system of the lamina was markedly affected. Injurious effects were most pronounced in the palisade parenchyma which shows that the different tissues respond differently to irradiation.

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The Gametophyte, Embryo, and Young Rhizome of *Psilotum triquetrum* Swartz

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With Plates, VIII and IX and sixty-seven Figures in the Text

INTRODUCTION

THE gametophyte generation of the Psilotaceae has been known for some twenty years. Lawson (1917a) first described the form and structure of well-grown gametophytes of *Tmesipteris tannensis*, including the sex organs and also one medium-sized embryo. He quickly followed up this paper with another (1917b) on the gametophyte of both *Tmesipteris* and *Psilotum triquetrum*. Darnell-Smith (1917) in the meantime had described certain features of the gametophyte of *Psilotum triquetrum*, recording also his successful attempt to germinate the spores. The present writer (1918) investigated *Tmesipteris tannensis* as it occurs in New Zealand, and described its gametophyte, embryo, and young sporeling. He later (1921) gave a fuller account of the embryo and sporeling.

The present paper is concerned with investigations on the life-history of *Psilotum triquetrum* carried out on the volcanic islet of Rangitoto, Auckland, N.Z.¹ During the course of a good many years the writer has paid three summer visits to this island, each of several days duration, during which he has obtained a large number of gametophytes of *Psilotum*. On the last occasion he was successful in finding the embryo and much also of the other material which forms the basis of this paper. In recent years Rangitoto Island has been under the control of a Domain Board, and the writer is glad to acknowledge his indebtedness to the Board for permission to camp on the island, and for allowing him the use of one of its huts.

THE OCCURRENCE OF PSILOTUM ON RANGITOTO ISLAND

This island, which rises symmetrically to a central extinct volcanic cone about 850 feet high, is some 5,000–6,000 acres in extent. It consists entirely of volcanic material. There is a peripheral belt, one or two hundred yards wide, with numerous large basaltic rocks interspersed with hollows and small gullies. The greater part of the island consists of scoriaceous lava. There is no running water, and the nature of the surface allows of no accumulation

¹ A preliminary note on this work has already appeared (Holloway, 1938).

[*Annals of Botany, N.S. Vol. III, No. 10, April 1939.*]

of rain-water whatsoever. The island is more or less closely covered with a sclerophyllous native shrubbery and low trees, but in the deeper hollows the vegetation is much more mesophytic, and includes most of the filmy ferns and other Pteridophyta which usually occur as epiphytes in typical Auckland wet forests.

Psilotum occurs in abundance in the peripheral belt, but is restricted to fissures in the large basaltic rocks, and other such exposed places, where often no other plants are to be found. Here not only are its subaerial parts subjected to great drying, which at times must be excessive, but this must also be the case with its rhizomes and gametophytes in the fissures and in the pockets of gritty soil. The soil completely lacks clay. Both Darnell-Smith (1917) and Lawson (1917b) state that in the neighbourhood of Sydney *Psilotum* grows abundantly in fissures on exposed sandstone cliffs, but that it also occurs commonly in permanently damp soil near waterfalls. Lawson also states that it is in damp soil of this kind that the gametophytes are more likely to be met with. The present writer, however, has found gametophytes in considerable numbers, both young and old, in various localities on Rangitoto Island where the soil can nowhere be permanently damp and is frequently dry. It is clear, then, that both the gametophyte and the sporophyte of *Psilotum* must possess in marked degree the ability to withstand the effects of drying.

On the other hand, *Tmesipteris* is certainly a mesophyte. On Rangitoto Island it occurs but rarely, and then only in deep shady hollows. It is a fairly common plant in wet forests throughout New Zealand, but not in exposed situations. Lawson also notes this characteristic difference between the two genera.

The writer made his first collection of *Psilotum* gametophytes during his second visit to Rangitoto Island, after a week's continuous search. Some seventy portions of mature large gametophytes were obtained from two dry pockets of soil which were over-hung by fertile plants. None of these portions had the basal end complete, some being withered off from behind, and others having been broken owing to the difficulty experienced in raking out the soil from the pockets. The special object of this visit was to obtain evidence of the embryo and sporeling, but careful examination of the objects under the microscope showed no attached embryos, nor did the soil yield young objects of any kind. Many of these gametophytes were found to be bearing gemmae in the manner described by Darnell Smith (1917).

The second collection was made some seven years later at a locality a few miles distant from that of the first. Here again a week's close search was made, with the result that large numbers of *Psilotum* objects of various types were found. These included young entire objects both gametophytic and sporophytic, and also well-grown gametophytes both of a slender and of a more robust form, the latter similar to those found previously. A number of attached embryos also were found.

All of the strongly growing gametophytes came, as on the previous occasion,

from pockets in which there was a considerable accumulation of soil, and into which spores could be shed from adjacent fertile plants. The smaller objects, including germinating gemmae, came for the most part from crevices in the faces of large blocks, in which also fertile plants were growing. These crevices were opened up by prizing off large flakes of the rock with a crowbar. The flakes, and also the sides of the crevices thus exposed, carry only a very thin film of humus on their surfaces, but many young *Psilotum* objects were found on these apparently dry surfaces, more especially inside the small cavities with which this type of rock abounds.

All soil obtained from the pockets and crevices was examined in water in a white plate, a very small portion at a time. Owing to the lack of clay it was thus comparatively easy, with the aid of a pocket lens, to see even such small objects as detached gemmae. The surfaces of the flakes were carefully brushed with water. Some of the soil was examined at once, but a large amount, after having been thoroughly soaked with water and drained, was taken back to the writer's laboratory at Dunedin and examined there.

THE DIFFERENT KINDS OF PSILOTUM OBJECTS

It is convenient to refer briefly at this point to the several different kinds of objects found.

Both the rhizome and the well-grown gametophyte are extremely brittle, and objects were not infrequently obtained from cramped pockets of soil as broken portions. Owing to the similarity in general appearance between such portions of the two generations, each object had to be closely examined for the presence or absence of sex organs. Both Lawson and the writer have noted this similarity in *Tmesipteris* also.

Again, it is known that gemmae, of closely similar form, are borne on both rhizome and gametophyte of *Psilotum*. Solms Laubach (1884) has described the sporophytic gemmae and their germination into young branching rhizomes, and has noted that the conducting-strand is differentiated very late in them. Darnell-Smith (1917) has recorded that they occur commonly on the gametophyte, but he did not trace their further germination.

Gemmae were found during the course of the present investigation in large numbers. Since mature sporophytes were usually present along with the gametophytes, the only thoroughly satisfactory method of following out the germination of either kind of gemma would be by means of experimental cultures. The writer has not attempted this, but from the study of the material found he is satisfied that gametophytes arising from gemmae are, previous to the formation on them of sex organs, in other words, up to a size of 1-2 mm., similar in both form and structure to young rhizomes arising from sporophytic gemmae. Similar objects of larger size, i.e. 4-5 mm. and upwards, if lacking sex organs, can confidently be regarded as sporophytes. Some scores of young *Psilotum* objects were obtained whose basal end showed that they were of gemma origin. The precise nature of some of the youngest of these

remained in doubt, others bore one or more sex organs, and others again could be regarded as sporophytes. The internal structure of these objects will be referred to later.

Young gametophytes which have originated from spores will best be distinguished from those which have had a gemma origin by the presence of the spore on the intact basal end. The writer has not been able to find the original spore on any of his gametophytes, either well grown or young. In contrast with this a good many of the small gametophytes of *Tmesipteris* which he had previously described, and also some of larger size, showed the original spore still attached (Holloway, 1921, p. 388). It is uncertain whether or not any of the *Psilotum* gametophytes found had originated from spores. The fact that so many of the small objects (bearing sex organs) showed a basal gemma indicates that this is the chief method of multiplication of the sex generation in the dry Rangitoto soil. Lawson (1917b) describes two of his *Psilotum* gametophytes as being entire, but his figures do not show the basal end intact. It cannot be said therefore whether they had originated from spores or from gemmae.

A young detached 'sporeling' rhizome, that is to say, one which has originated as an embryo, can, in external view, be determined by the presence on it of the foot-scar. A young rhizome of gemma origin can be determined in external view either by the presence of the originating gemma or by the distinct tapering of the basal end of the rhizome. A good many entire young *Psilotum* objects, a few millimetres or more in length, were found which, lacking sex organs, could safely be regarded as developing sporophytes. Most of these could be distinguished either as sporelings, or as gemma-rhizomes respectively, by one or other of the above-mentioned features. A further point of distinction was apparent after the objects had been sectioned. In the sporeling rhizome the vascular strand of the axis originates in the close vicinity of the foot, whereas in the gemma-rhizome its origin is delayed.

THE GAMETOPHYTE—MATURE FORM

The mature gametophyte of *Psilotum* (Text-figs. 1–6) has the same general form as that of *Tmesipteris*. It is an altogether subterranean, elongated, cylindrical, branching body, bearing numerous stiff brown rhizoids. Its colour, which is due partly to the endophytic fungus, and partly to the cutinization of the superficial cell-walls, is dark brown in the older parts, a more clear brown farther forward, and clear at the growing apices. Branching takes place by forking of the apex (Text-figs. 1 and 2). During elongation there is a more or less progressive widening of the apex, this being best seen in young gametophytes, and hence also in the oldest parts of those of greater age (Text-figs. 4 and 5).

The largest found were from 12 to 18 mm. in length and from 1 to 2 mm. in diameter (Text-figs. 3, 44, 45, 48). None of these were complete, the hinder ends of all well-grown specimens being either broken or decayed.

Lawson states (1917b, p. 100) that the gametophytes of *Psilotum* found by him were larger and thicker than those of *Tmesipteris*, the largest of the former, which was, however, not complete, being about 18 mm. long and a little under 1 mm. in diameter. Some of the gametophytes of *Tmesipteris* described by the present writer (1921, p. 390) were as long as and considerably thicker than those of *Psilotum* described by Lawson, and some of the portions of *Psilotum* gametophytes from Rangitoto Island were still thicker.

While actually engaged in dissecting the soil the writer noticed that there were two main sizes of the *Psilotum* gametophyte, viz. a slender and a robust size. Gametophytes of the latter type (Text-figs. 3, 22, 44, 45, 48) were obtained from several localities and always from pockets of soil. Those of the slender form (Text-figs. 4, 5, 18, 20), some of which showed at the basal end the gemma from which they had grown, were obtained in considerable numbers from the comparatively bare surfaces of levered-off flakes of rocks. The seventy portions found during the first collection were all of the robust form. It was also very noticeable that the antheridia on these larger gametophytes (Text-fig. 38) were much larger than those on the slender form (Text-fig. 39), and the same difference was afterwards found to hold for the archegonia. It must be added, however, that intermediate sizes of the sex organs were also afterwards found. After a large number of gametophytes of the two sizes had been sectioned, a third difference between them became apparent, namely, that many of the thickest specimens possessed a well-defined vascular strand which was quite absent from those of slender form. If it were not for this latter feature the difference in size might be assumed to be a matter of age only.

GAMETOPHYTIC GEMMAE AND BUDS

Reference has already been made to the description given by Solms Laubach (1884) of sporophytic gemmae, and to the fact that Darnell-Smith (1917) has recorded them also on the *Psilotum* gametophyte.

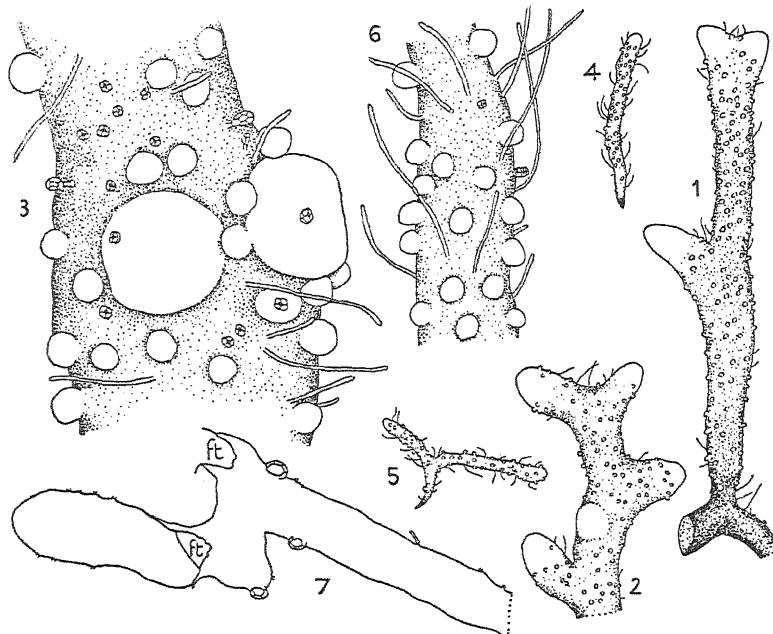
The writer on his first visit to Rangitoto Island collected gemmae from mature rhizomes, a few of which are illustrated in Text-figs. 8-12, for comparison with those derived from gametophytes. Some were found actually attached to the rhizomes, and, since no gametophytes were discovered on this occasion, it can be assumed that all of them were of rhizome origin.

The gametophytes obtained on each of the two subsequent visits bore gemmae similar to those on rhizomes, and sometimes in large numbers. They were found even on the youngest gametophytes which were themselves of gemma origin.

In Text-figs. 13-15 are shown three gemmae which were actually attached to gametophytes. There is nothing to distinguish between gemmae from gametophytes and those from rhizomes. Many detached, and also germinating gemmae (Text-fig. 16) were obtained by carefully brushing the surfaces of the rock flakes, but since both gametophytes and sporophytes were here

present the precise nature of such gemmae was uncertain. The object shown in Text-fig. 17 has come from a gemma and bears one antheridium: in this case, then, the gemma is gametophytic.

Gemmae do not occur on either the rhizomes or the gametophyte of *Tmesipteris*. Both Lawson and the writer have had ample opportunity for finding them if they had been present. On the other hand, vegetative 'buds',

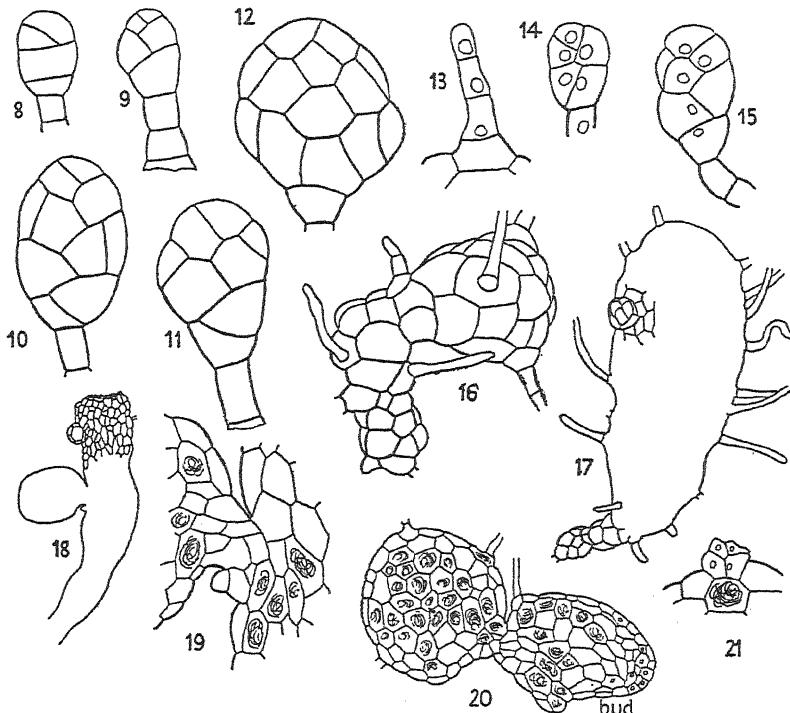


TEXT-FIGS. 1-7. Figs. 1-2. Portions of well-grown gametophytes covered with antheridia. $\times 4$. Fig. 3. Portion of extra large gametophyte showing sex organs and three embryo protuberances. $\times 16$. Figs. 4-5. Two practically entire gametophytes of slender build. $\times 4$. Fig. 6. Portion of well-grown gametophyte with antheridia. $\times 16$. Fig. 7. A slender gametophyte bearing a young sporeling and also the foot of another. $\times 16$.

of a very different manner of origin, but of a similar function to that of the gemma, occur occasionally on the gametophyte in both genera. The writer (1921) has already described them for *Tmesipteris*. The gemma originates from the terminal cell of a (usually) three-celled rhizoid-like hair. The bud (Text-fig. 21) arises directly from a single, or from two, surface cells which at once subdivide and protrude.

In *Tmesipteris* the bud early develops an apical growing cell, but this stage was not observed in *Psilotum*. In the latter plant buds were found on only three gametophytes, all of which were of the slender form. Further growth (Text-figs. 18-20) results in the formation of a more or less globular body with an apical growing cell. By this time it is already infected by the fungus. At this stage it bears a misleading likeness to a protruding embryo,

but serial sections through the point of attachment reveal at once its real nature. Its further development was not followed, as was the case also in *Tmesipteris*. It is possible that some of the gametophytes collected had originated as buds.

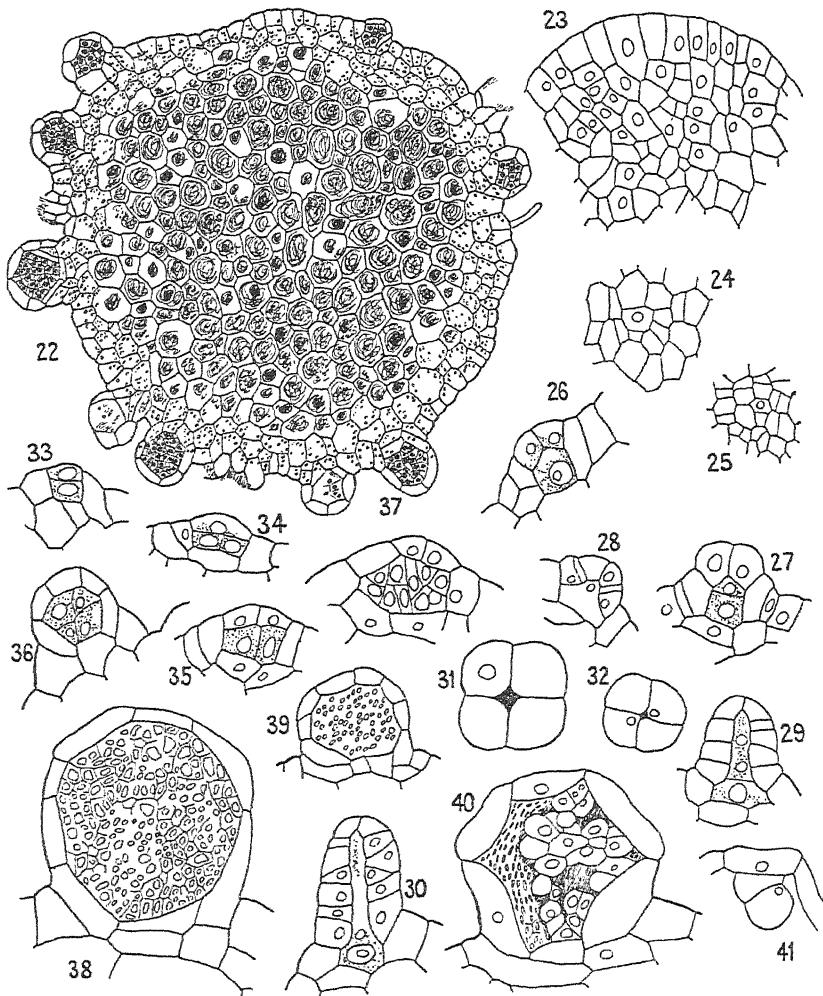


TEXT-FIGS. 8-21. Figs. 8-12. Sporophytic gemmae. $\times 137$. Figs. 13-15. Young attached gametophytic gemmae. $\times 137$. Fig. 16. Germinating gemma either sporophytic or gametophytic. $\times 68$. Fig. 17. Young gametophyte of gemma origin bearing an antheridium. $\times 34$. Fig. 18. Vegetative bud on a slender gametophyte. $\times 17$. Fig. 19. Median section through the point of attachment of bud shown in Fig. 18. $\times 68$. Fig. 20. Median section through the point of attachment of another bud. $\times 34$. Fig. 21. A very young bud. $\times 68$.

THE GAMETOPHYTE, GENERAL STRUCTURE

The general structure of the mature gametophyte of *Psilotum* is similar to that of *Tmesipteris*, with the exception that in certain cases the former possesses conducting tissues. A median longitudinal section of the forward forking end of a large gametophyte is shown in Pl. VII, Fig. 1. In this figure antheridia and archegonia, conducting tissues, and the endophytic fungus can all be seen.

Except for the growing apices, and for a one- or two-layered peripheral zone carrying the sex organs, the fungus is distributed uniformly throughout the tissues. In those cases in which conducting tissue is present the fungus



TEXT-FIGS. 22-41. Fig. 22. A well-grown gametophyte in transverse section showing numerous antheridia. $\times 50$. Fig. 23. Apex of gametophyte in median longitudinal section showing segmentation of the apical cell. $\times 100$. Figs. 24-5. Apices of two gametophytes in transverse section showing the four-sided apical cell. $\times 100$. Figs. 26-30. Stages in development of the archegonium. $\times 150$. Figs. 31-2. Transverse sections of the necks of two archegonia borne on a large and a slender gametophyte respectively. $\times 150$. Fig. 33. Young sex organ of indeterminate nature. $\times 150$. Figs. 34-7. Stages in development of the antheridium. $\times 150$. Figs. 38-9. Two mature antheridia borne on a large and a slender gametophyte respectively. $\times 100$. Figs. 40-1. Abnormal antheridia showing proliferation of the wall cells. $\times 100$.

is absent also from the axial region. Text-figs. 20 and 22 show slender and robust gametophytes in transverse section. Starch is usually present in the case of the large gametophytes in the apical and peripheral fungus-free regions, and especially in the zone around the axial strand.

In the older parts the fungus is for the most part clumped in the cells, but farther forward it takes the usual form of dense skeins. In the more forward regions of gametophytes, more especially of those of large size, the ends of the hyphae in the cells are commonly greatly dilated, forming vesicles, several such being present in each cell. These vesicles are especially noticeable in longitudinal sections.

The gametophyte grows by means of a single apical cell which has four cutting faces. This is shown in longitudinal view in Text-fig. 23. In Text-figs. 24 and 25 is seen in transverse section the segmentation of the apical cell of two gametophytes.

THE ARCHEGONIA AND ANTERIDIA

The sexual organs are scattered over the entire surface of the gametophyte and are intermixed. As Lawson has noted (1917b), there are much fewer archegonia than antheridia. In *Tmesipteris*, on the other hand, the archegonia are much more numerous. This characteristic difference between the two genera may possibly be related to their different habitats. The portion of a large gametophyte represented in Text-fig. 3 has an unusually large number of archegonia in view.

The young sex organs are to be met with mainly on the apical regions, but occasionally young antheridia are to be found on older parts. At the two-celled stage (Text-fig. 33) it is not possible to distinguish between the two organs. A number of both kinds were met with at the three-celled stage, the inner of the two cells having divided in the case of the antheridium (Text-fig. 34), and the outer in the case of the archegonium (Text-fig. 26). At this stage, then, they are readily distinguished.

The primary neck cells of the archegonium, after having divided again to form the four-celled rosette, begin to project strongly owing to the growth of the inner cell, and the latter then divides to form the primary neck canal cell and a lower cell (Text-fig. 27). No basal cell is formed. The projecting neck cells now begin to divide transversely to give rise to the neck tiers (Text-fig. 28). In *Tmesipteris* the writer was unable to demonstrate how many neck canal cells were formed. Lawson does not give details as to the axial row in either genus. A number of developing archegonia were met with in the present study in which the egg and ventral canal cells were clearly to be seen, but not the neck canal nuclei (Text-fig. 30). In two other cases of rather younger age two nuclei were present in the neck canal. In one of these (Text-fig. 29) it is not clear whether or not both of these nuclei represent neck canal cells. In the second case the two nuclei were both situated near the upper end of the canal, and hence were probably neck canal cells. The

mature archegonium (Text-fig. 30), like that of *Tmesipteris*, has a perfectly straight neck. The maximum number of neck tiers is six or seven.

As Lawson states (1917b), there is some variation in the way in which the neck tiers break away in the mature archegonium. In some cases it breaks off more or less flush with the surface of the gametophyte, this being also the normal method in *Tmesipteris*. Frequently, however, a less number of tiers break off, and the writer has seen old archegonia on the hinder parts of gametophytes in which the neck is still complete. This is probably due to the fact that the cutinization of the superficial cell-walls of the gametophyte is less marked in *Psilotum* than in *Tmesipteris*, a difference which would not be expected from a comparison of their respective habitats.

Some early stages in the development of the antheridium are represented in Text-figs. 34–7, and also two mature antheridia in Text-figs. 38 and 39. The mature antheridium projects strongly from the surface, as in *Tmesipteris*, but occasionally one was seen in which this is not so marked (Text-fig. 37).

The two antheridia shown in Text-figs. 38 and 39 are from gametophytes of a robust and a slender form respectively. The writer has measured a considerable number of mature antheridia on such gametophytes and can say that the two figured are typical. Moreover, there is a similar difference in the size of the archegonia. In Text-figs. 31–2 are shown in transverse section the necks of typical archegonia borne by the large and the small types of gametophyte respectively.

Lawson's statement (1917b, pp. 104–6) that the sex organs of *Psilotum* are very much smaller than those of *Tmesipteris* does not hold as a general rule. A comparison of his figures with those of the present writer shows a considerable variation in the size of the sex organs in both genera. Lawson's descriptions and figures of the *Psilotum* gametophytes make it clear that they were not as robust as some of those described in the present paper, and the dimensions of their sex organs agree more or less with those of the present writer's slender form of gametophyte.

GAMETOPHYTIC CONDUCTING TISSUES

About thirty of the largest portions of gametophytes found, some of these belonging to the first collection and others being obtained some seven years later from a widely distant locality, were found to possess a well-defined conducting strand. Lawson makes no reference to any conducting tissue in the gametophyte of either *Psilotum* or *Tmesipteris*, nor has the present writer found it in the latter.

The description which follows may first be summarized. The strand originates at the apex; it is discontinuous, fading out and reappearing usually several times in the length of a few millimetres; it has a distinct limiting endodermis; and in regions of the gametophyte well behind the apex it commonly possesses 1–3 centrally placed annular or scalariform tracheids.

In Text-fig. 42 is shown the apex of a large gametophyte in median longi-

tudinal section in which a slender conducting strand is commencing to form immediately behind the actual apex. A considerable number of apices were sectioned longitudinally, and the apical origin of the strand was seen in three cases.

In Text-fig. 43 is the apical region of another large gametophyte, also in median longitudinal view, in which the apex is seen to be in a more or less inactive condition. Careful examination of the neighbouring sections in this series failed to locate an actual apical cell. The fungus has here extended forward and has occupied the tissue in advance of the strand. In many of these forward cells the fungal coils are in a clumped condition indicating that the apex had not recently been active. Moreover, two old antheridia are present close behind the apex. This condition of the apical region was observed in several cases, and it is clear that the apex is intermittently active. When active it gives rise to the strand, and when comparatively inactive the fungus is able to advance closer up to the actual apex than usual. Starch is present in the foremost cells of the strand. Compare also the apex shown in Pl. VIII, Fig. 1.

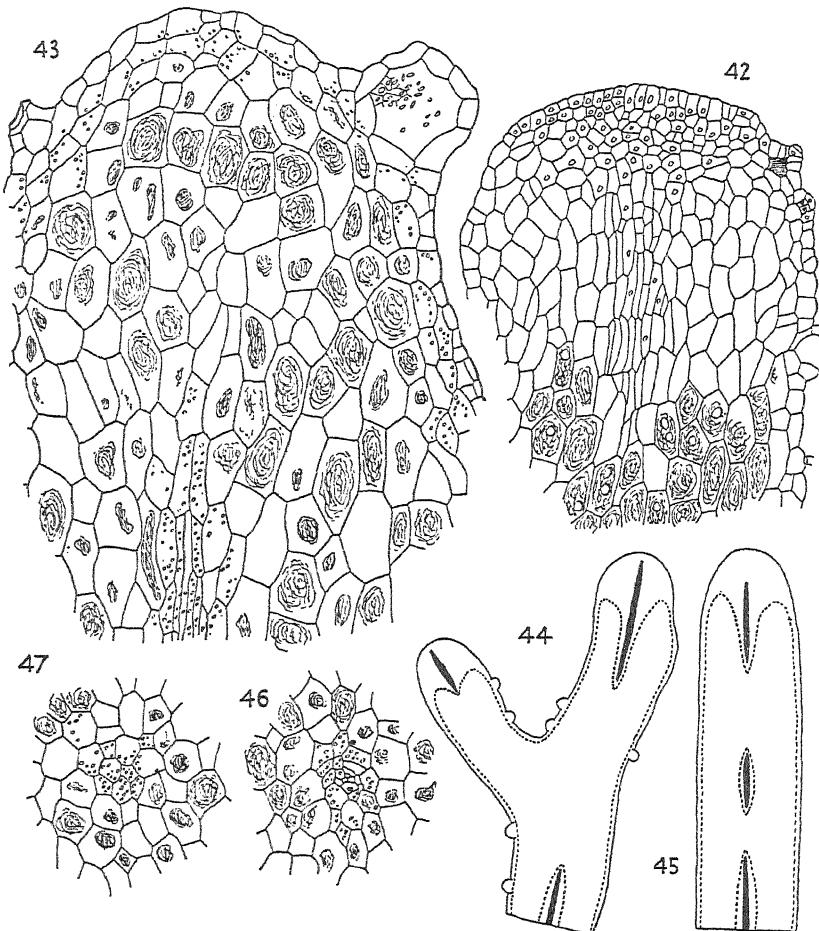
Diagrams of the forward portions of two large gametophytes in median longitudinal view are given in Text-figs. 44 and 45. The first of these objects was cut longitudinally, and the second was cut transversely throughout its complete length. The diagrams are drawn to scale and illustrate the intermittent course of the strand, and also the distribution of the fungus. The general appearance of the axial strand in longitudinal view is seen in Pl. VIII, Fig. 2.

In Text-figs. 46 and 47 is the central region of a gametophyte at a place where the strand is about to fade out. Where it is still present (Text-fig. 46) its cells are free from both starch and fungus, and it is surrounded by a fungus-free starch zone. Where it is about to fade out the central cells contain starch (Text-fig. 47). Beyond this point the fungus extends right to the centre (see also Text-fig. 43).

A well-formed conducting strand, but lacking tracheids, is illustrated in Text-fig. 48 in a complete transverse section of a large gametophyte. Both antheridia and archegonia are also in view. Another feature is the fungus-free starch zone. The cutinization of the radial walls of the endodermal cells is well seen when stained with safranin. This feature is shown on a larger scale in Text-fig. 49. Single microtomed and hand-cut sections were also tested with phloroglucin which stained the cutinized parts of the endodermal walls and also tracheides brilliantly. In all well-formed strands examined an endodermis was found to be present whether the strand was old enough to possess tracheids or not.

Tracheids were present in sixteen out of the thirty portions of large gametophytes which were sectioned. These were cut serially either transversely or longitudinally and were stained with safranin and Delafield's haematoxylin. Although it is possible that these sixteen objects do not represent as many

distinct gametophytes, some being broken when collected, nevertheless it is apparent that the occurrence of tracheids is not an unusual feature. A transverse section of a large gametophyte is shown in Pl. VIII, Figs. 3 and 4,



TEXT-FIGS. 42-7. Fig. 42. Median longitudinal section of apex of large gametophyte showing apical origin of conducting strand. $\times 50$. Fig. 43. Median longitudinal section of apex of another gametophyte showing apex in inactive condition. $\times 50$. Figs. 44-5. Diagrams drawn to scale of the forward portions of two large gametophytes in longitudinal view, showing the discontinuous conducting strand and also the distribution of the fungus. $\times 10$. Figs. 46-7. The axial region of a large gametophyte in transverse section showing fading out of the strand. $\times 50$.

and a median longitudinal section in Pl. IX, Figs. 5 and 6. A well-formed strand possessing tracheids is present in both cases, and also sex organs at the surface.

When viewed in transverse sections the tracheids are seen to be from 1 to 3 in number, and are invariably centrally placed (Text-fig. 50 and Pl. VIII, Fig. 4). In Text-fig. 52 is shown in longitudinal view a strand with a single tracheid. The narrow, thin-walled cells which immediately surround the tracheid are much elongated with tapering ends, and contain densely staining cytoplasm and large nuclei. Careful search was made for sieve areas on their walls, but such were not found. The tracheids are also much elongated, with blunt or tapering ends and have either closely spaced annular thickenings (Text-figs. 52 and 53) or are scalariform (Text-fig. 54 and Pl. IX, Fig. 6).

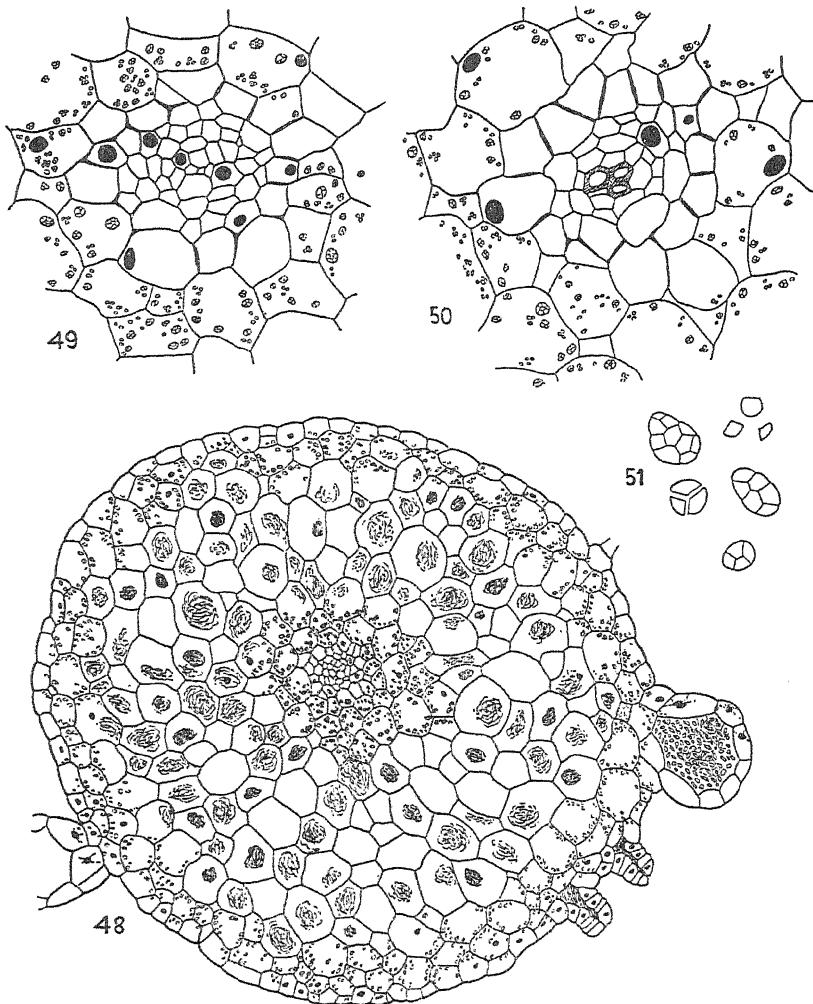
In the hinder parts of a few large gametophytes a bright-brown irregular deposit of phlobaphene was observed on the walls of occasional cells close outside the strand. This is a well-known and characteristic feature in the mature rhizome of both *Psilotum* and *Tmesipteris*. In a few other cases one or more elongated dead cells with thickish brown walls were observed in strands in which tracheids were lacking. Under the oil immersion objective it was apparent that the walls had been thickened with a brown deposit which did not have the same appearance as phlobaphene.

Particular attention has been given to determining whether or not the strand-bearing gametophytes possess any other unusual features. Except for their larger size they are similar in form to those which lack a strand. They bear sex organs in the usual large numbers. That these organs are functional is indicated by the fact that healthy well-grown embryos were borne by three such gametophytes.

As has already been described, the sex organs in these cases are especially large. In three strand-bearing gametophytes a few abnormal antheridia were observed, in one case twelve, in which proliferation inwards of one or more of the wall cells had taken place leading to crushing of the antheridial contents. An example of this is shown in Text-fig. 40. In Text-fig. 41 it is seen that the ingrowing cells arise from the wall of the antheridium.

The large gametophytes differ markedly from those of the slender form, and also from those of *Tmesipteris*, in the abundant accumulation of starch, which takes place both in the peripheral zone and also in the close neighbourhood of the conducting strand. It may be noted here that the grains are large and compound (Text-fig. 51), the same type of grain being characteristic of gametophyte and sporophyte in both *Psilotum* and *Tmesipteris*.

Careful measurements have been made of all the strand-bearing gametophytes sectioned in order to determine the relation between the presence of the strand and the size of the gametophyte. The thirty objects in which a strand was found were all over 1 mm. in diameter, twenty-four of them having a diameter of from $1\frac{1}{2}$ to 2 mm. The slender gametophytes shown in Text-figs. 4, 5, 18, and 20 have a diameter of about $\frac{1}{2}$ mm. In several portions of large gametophytes from 3 to 6 mm. in length and 1 to $1\frac{1}{2}$ mm. in width no strand at all was found. It would thus appear that the strand makes its



TEXT-FIGS. 48-51. Fig. 48. Transverse section of a large gametophyte in a region where a well-formed conducting strand is present, with a limiting endodermis, but lacking tracheids. $\times 50$. Fig. 49. Transverse section of a gametophytic conducting strand similar to that in Fig. 48. $\times 160$. Fig. 50. Transverse section of a gametophytic conducting strand showing three tracheids. $\times 160$. Fig. 51. Compound starch grains from a gametophyte. $\times 480$.

first appearance at a growing apex when the progressively widening gametophyte has attained a diameter of at least 1 mm.

THE EMBRYO

No embryos were found on the seventy portions of large gametophytes which comprised the first collection. These came from two dry pockets of

soil in which opportunities for fertilization would probably have been rare. A large number of these objects were sectioned: the remains of an old embryo foot was found in one of them.

From the second collection thirty embryos were obtained which illustrate the essential features of the development. Twelve of these embryos were borne on one gametophyte, and ten on another, a portion of the latter being shown in Text-fig. 3. In each of these cases the gametophyte possessed a well-formed conducting strand. In these two cases most of the embryos were very young. The soil from which they came was dissected out in the laboratory, having been by that time in a well-moistened condition for two to three weeks. It is likely that fertilization here had taken place subsequently to the actual collection in the field. This indicates a simple method of obtaining embryo stages in subterranean gametophytes. It is apparent that apart from these youngest stages not many were obtained considering the large number of gametophytes found. It would seem that opportunities for fertilization are infrequent in the Rangitoto rock crevices.

In the general sequence of segmentation, and in the structure of the embryo at the different stages of development, there is a very close similarity between *Psilotum* and *Tmesipteris*.

The first division of the fertilized egg is by a wall transverse to the axis of the archegonium (Text-figs. 55 and 56). This divides the embryo into its two body organs, the epibasal cell giving rise to the shoot, and the hypobasal cell to the foot. No root or cotyledon is formed. This transverse basal wall afterwards persists as a distinct line of demarcation between shoot and foot, and it is here that the young rhizome eventually separates away from the foot, leaving the latter embedded in the gametophyte.

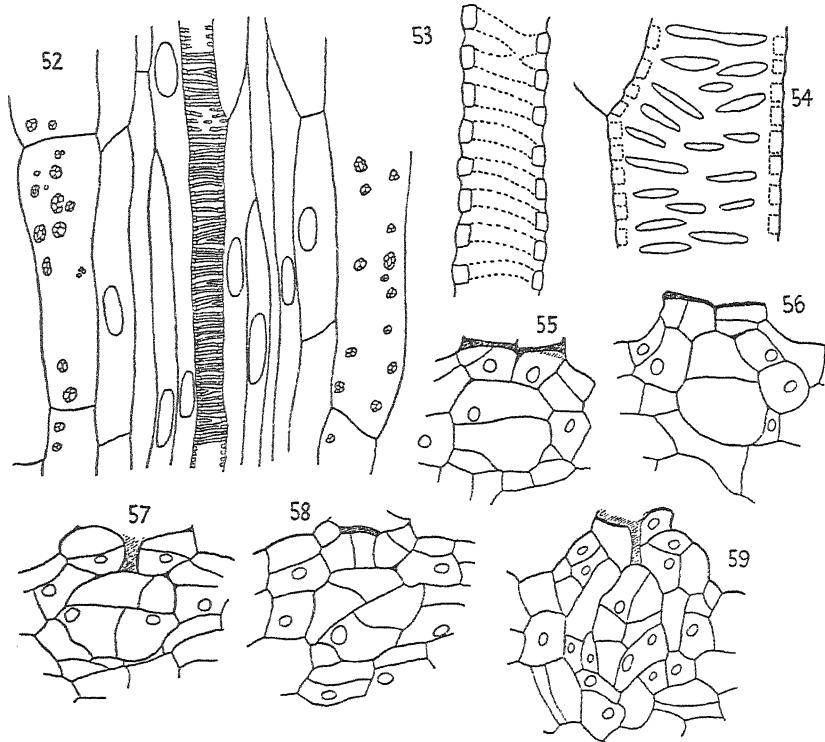
The four-celled stage is attained by each of these two cells dividing by a more or less inclined wall (Text-figs. 57 and 58). The subsequent sequence of segmentation is for a time irregular. In the embryo shown in Text-fig. 59 cell division has been confined to the epibasal segment, whereas in that shown in Text-fig. 60 the hypobasal or foot segment has been more active.

The embryo in Text-fig. 61A is not shown quite medianly, and the archegonial neck does not appear in the figure. The section selected for illustration shows well the basal wall and also the beginning of the haustorial outgrowth of the peripheral cells of the foot. An apical cell had already been set apart near the upper end of the shoot (Text-fig. 61B).

Text-fig. 62A illustrates a later stage in the development, in which the shoot is about to break through the calyptra. This embryo was borne on a large gametophyte and has a particularly well-developed foot. Unfortunately the shoot was damaged, and so is not figured in detail. The original basal wall is distinct. Text-fig. 62B represents a tangential section through the foot of the same embryo, and shows the extensive development of the haustorial outgrowths.

A protruding embryo, borne on a slender gametophyte, is shown in median

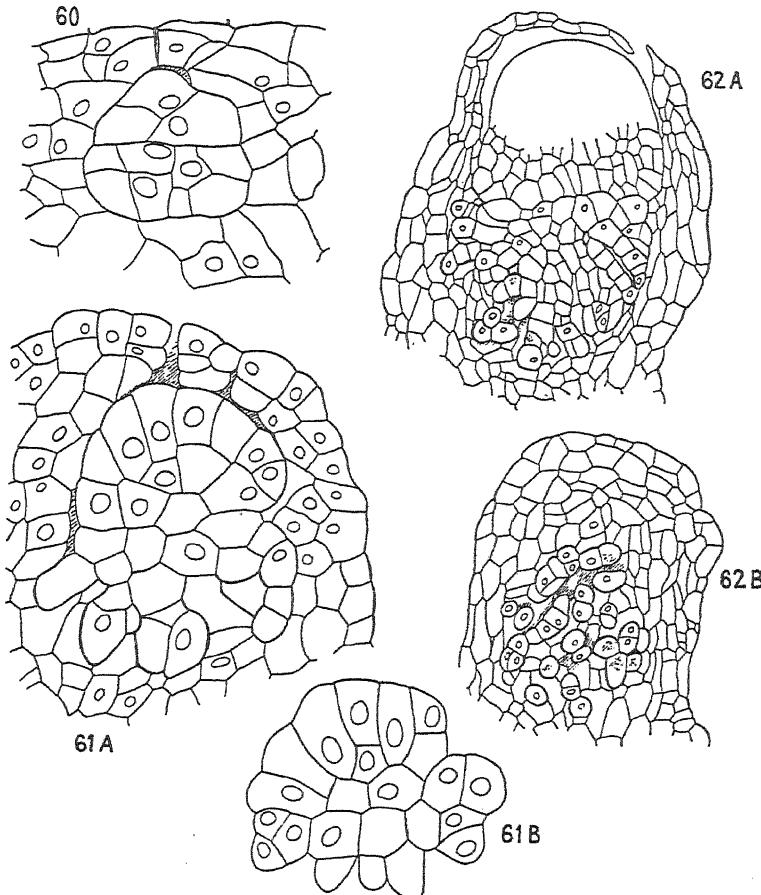
longitudinal section in Text-fig. 63, the section missing the apical cell of the shoot. The foot is relatively small. The shoot has not yet become infected by the fungus, and there is no accumulation of starch in shoot or foot or gametophyte.



TEXT-FIGS. 52-9. Fig. 52. A gametophytic conducting strand in longitudinal section showing one annular tracheid. $\times 192$. Fig. 53. Portion of an annular tracheid on larger scale. $\times 850$. Fig. 54. Portion of a scalariform tracheid. $\times 850$. Figs. 55-6. Two embryos at the two-celled stage in median longitudinal section. $\times 137$. Figs. 57-8. Two embryos at the four-celled stage in median longitudinal section. $\times 137$. Fig. 59. A slightly older embryo in median longitudinal section. $\times 137$.

A much older, but still attached embryo, or sporeling as it can now be termed, is shown in median longitudinal view in Text-fig. 64A. This also was borne on a slender gametophyte. The foot, which is cut somewhat obliquely, was not large. The main apex and conducting strand is in median view, the latter leading to the foot. The shoot is infected with the fungus which extends well forward towards the apex. In the *Tmesipteris* embryo a second growing-point is not infrequently present in the shoot by the time that the latter has begun to protrude from the calyptra, the shoot thenceforward elongating in two directions. In the sporeling shown in Text-fig. 64A a second apex was present at the base of the main axis, and had elongated

slightly in a direction inclined at an angle to that of the latter. This is indicated in Text-fig. 64B, which represents another section through the base of the embryo, missing the foot, but showing the strand which leads to the second apex.



TEXT-FIGS. 60-62B. Fig. 60. A young embryo in median longitudinal section showing rapid segmentation of the foot. $\times 137$. Fig. 61A. A still older embryo, cut somewhat obliquely, showing haustorial nature of the foot. $\times 137$. Fig. 61B. Another section of the same embryo showing the apical cell of the shoot. $\times 137$. Fig. 62A. A well-grown embryo in median longitudinal section about to burst through the calyptra. The shoot was damaged. $\times 68$. Fig. 62B. A tangential section through the foot of the same embryo, showing haustorial outgrowths. $\times 68$.

A few instances were observed in which a foot, from which the shoot had become detached, was embedded in the tissues of the gametophyte. One of these is illustrated in Text-fig. 65, and it is apparent that the detachment has taken place along the line of the original basal wall. On one detached

sporeling (Text-fig. 66) the entire foot, with a fragment of the surrounding gametophytic tissue, was present. So far as the writer's observations go this is an unusual occurrence.

THE YOUNG SPORELING RHIZOME AND GEMMA RHIZOME

A considerable number of entire young rhizomes, 3 or 4 to 10 mm. in length, were found, and it was necessary to determine whether these had originated from embryos or from sporophytic gemmae. They were not gametophytes as shown by the absence of sex organs. As has already been mentioned, a rhizome of this size, which has a tapering basal end, and on which there is no foot scar, can be regarded as of gemma origin. The late origin in it of the conducting strand will indicate the same thing. An entire sporeling rhizome, on the other hand, will show the scar of the foot, and, on being sectioned, will also show the conducting strand leading into the immediate neighbourhood of the foot.

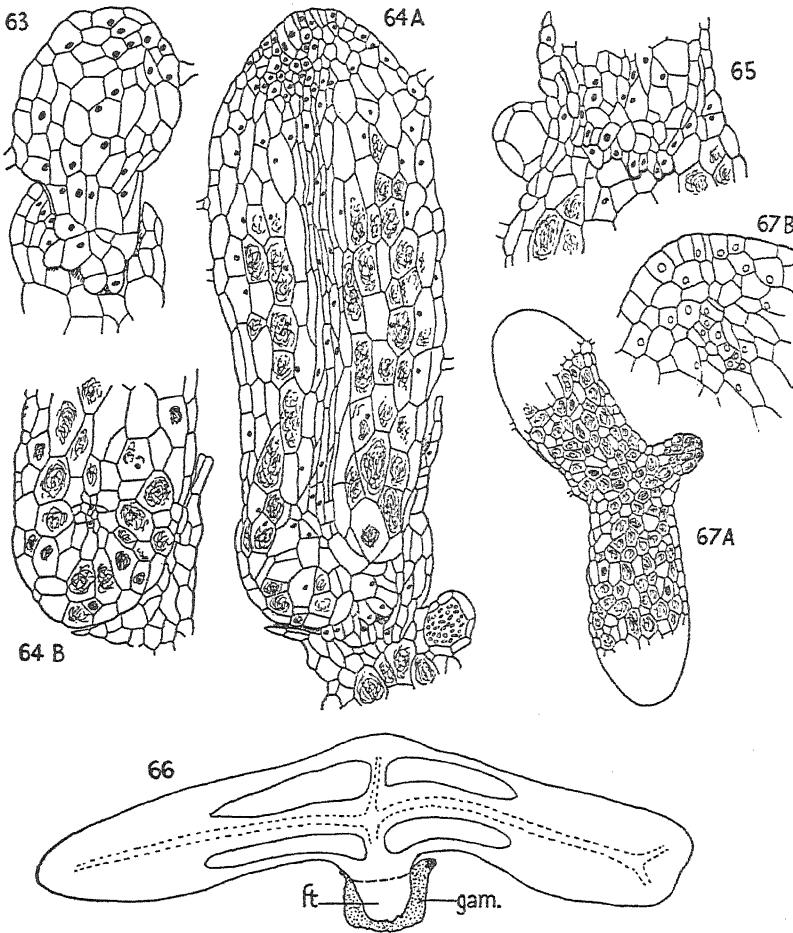
Text-fig. 66 is a diagram drawn to scale of an entire detached sporeling rhizome, 4 mm. in length, in median longitudinal view. There are two growing apices, one of which has recently forked, the conducting strands of the two halves of the axis leading towards the foot. The distribution of the fungus is also shown. A third apex is present on the side of the shoot opposite to the foot, and a slight strand of conducting tissue leads towards it from the main strand.

For comparison with this, a median longitudinal section of an entire young rhizome of gemma origin is given in Text-fig. 67A. The two apices are not in median view, but one of them is shown in another section in Text-fig. 67B in which a slight delayed strand has recently been formed. The absence of conducting tissue from the main body of this object shows that it is not a sporeling. The lateral protuberance is probably the originating gemma. Several somewhat older entire rhizomes of gemma origin, 5–9 mm. in length, were sectioned serially. The precise nature of these objects was indicated by the features mentioned above. It was found that the conducting strand was lacking in the more hinder parts of these rhizomes, but that when once it is initiated it is continuous as in the sporeling rhizome, and is not intermittent as is the case with the gametophytic strand.

No sporeling or gemma rhizomes were found which illustrate the further development into the mature sporophyte. Presumably this will be similar to what takes place in *Tmesipteris* where, after a period of altogether subterranean elongation and branching, one or more of the branches emerge from the soil as 'leafy' shoots.

DISCUSSION

Lawson (1917b) showed that the gametophytes of *Psilotum* and *Tmesipteris* closely resemble one another in general form and structure and in the structure of the sex organs, and the observations of the present writer have



TEXT-FIGS. 63-67B. Fig. 63. A protruding embryo borne on a slender gametophyte, in median longitudinal section. $\times 68$. Fig. 64A. The attached young sporeling shown in Fig. 7, in median longitudinal section: the foot is cut obliquely. $\times 50$. Fig. 64B. Another section through the base of the same sporeling, showing a conducting strand leading to a second apex. $\times 50$. Fig. 65. The foot of detached sporeling, embedded in a gametophyte. $\times 68$. Fig. 66. A diagram drawn to scale of a detached sporeling, showing three apices, conducting strands, and the distribution of the fungus. The entire foot is also present. $\times 22$. Fig. 67A. A young rhizome of gemma origin in median longitudinal section showing the originating gemma. The apices are not cut medially. $\times 22$. Fig. 67B. One of the apices of the same object, in somewhat oblique section, showing late origin of conducting strand. $\times 95$.

confirmed this. There are several particulars in which they differ, but only one of these, viz. the presence of a vascular strand in the gametophyte of *Psilotum*, need be discussed here in detail. Whether or not the characteristic gametophytic features which they possess in common can be held to be due

to the saprophytic and subterranean habit, the fact remains that with respect to a number of them the two genera differ from all other saprophytic Pteridophyta. The same can now be said also of the embryo and sporeling. Thus the complete knowledge of the life-history of the two genera has confirmed the opinion, originally based upon the study of the sporophyte generation alone, that they constitute a clearly defined natural group.

The explanation of the occurrence of vascular tissues in the largest gametophytes of *Psilotum* is not obvious, and the different interpretations possible raise questions of wide significance.

It has been shown above that these specially robust gametophytes possess certain other peculiar features in addition to the vascular strand, as for example sex organs of an extra large size. On account of their peculiarities it might be supposed that they are in some way inherently different from the non-vascularized gametophytes. There are two modes of origin possible for the *Psilotum* gametophyte, viz. from spores and from gemmae (and buds) respectively. Since, however, both spores and gemmae belong to the same (haploid) generation, there seems to be no reason to suppose that this difference in origin is connected in any way with the subsequent differences in structure. Again, there is the possibility of some derangement of the normal nuclear cycle in *Psilotum*, resulting in a diploid or heteroploid condition in the gametophyte. It must be stated at once that the writer has not made the cytological examination necessary to settle this point. Such an abnormal condition would presumably affect all the gametophytes and not merely certain individuals. Moreover, the vascularized gametophytes seem to be normal, judging from the fact that they bear the usual crop of antheridia and archegonia, and that these organs are functional as shown by the presence of healthy embryos on such gametophytes. The occurrence of a few abnormal antheridia on some of these large gametophytes is probably not of general significance. The vascularized gametophytes were found at two widely separated localities; the second occasion some seven years later than the first.

A second explanation possible is that physiological changes take place in the *Psilotum* gametophyte after it has attained a certain size. With regard to size, as indicated by diameter, the writer has made a careful examination of Lawson's figures and descriptions of the gametophytes of both *Tmesipteris* and *Psilotum*, and of his own corresponding material and figures. So far as these descriptions go it is clear that the presence of the conducting strand in *Psilotum* is correlated with special robustness of growth. Lawson does not record the diameter of the gametophytes of *Tmesipteris* found by him, but from his figures (1917b) this apparently does not exceed 0.6 mm. The greatest diameter in this genus observed by the present writer (1921) was 1.25 mm. No trace of gametophytic conducting tissue has been found in *Tmesipteris*. With respect to the *Psilotum* gametophyte, Lawson's largest specimens were well under 1 mm. in diameter (1917b, fig. 19), and here also he does not mention a conducting strand. The strand-bearing gametophytes of *Psilotum* found by

the present writer, thirty in number, were all over 1 mm. in diameter, the majority of them ranging from $1\frac{1}{2}$ to 2 mm. Although this material did not provide an instance of the actual beginning of the strand in an entire developing gametophyte, nevertheless the above data indicate that it makes its first appearance in the progressively widening apex when this has attained a diameter of at least 1 mm.

It is apropos to note here that the figures and descriptions given by several writers of the elongated subterranean gametophytes of other Pteridophytes show that the occurrence of an axial tissue having a conducting function is not an unusual phenomenon. Lang (1902) has recorded that in both *Ophioglossum pendulum* and *Helminthostachys* the axial cells are somewhat elongated. In the case of the former he states that in the older basal regions of the gametophyte the fungal endophyte is present right to the centre, but that farther forward it disappears from the axial cells. In *Helminthostachys* the appearance of the axial tissue, he states (p. 35), 'suggests that it is useful in the conduction of plastic material'. Campbell (1911) makes corresponding statements in his descriptions of these same gametophytes. Again, the present writer (1920) has described a distinct axial tissue consisting of considerably elongated fungus-free cells in the main axis and branches of the gametophytes of the epiphytic *Lycopodium Billardieri*.

In these cases the significance of the conducting tissue is no doubt physiological only. In the *Psilotum* gametophyte the strand is of a much more complex nature, possessing lignified annular or scalariform tracheids and a limiting endodermis. It is possible that the same explanation holds here also, namely that the strand, as also the other special features which accompany it, such as the large size of the sex organs, the abundant accumulation of starch, and the occasional abnormal structure of the antheridia, are of physiological significance only. Such an explanation, however, in the case of *Psilotum* raises an important question, since it involves the possibility that lignified tracheids and an endodermis, hitherto regarded as peculiar to the sporophyte generation, can arise in the gametophyte in response to special physiological conditions.

A third, quite distinct, interpretation of this gametophytic strand in *Psilotum* is that it is vestigial and has phyletic significance. Both Lawson and the present writer have remarked upon the striking similarity in external appearance between the gametophyte and the young sporophyte in the Psilotaceae. To some extent, at least, this similarity may be held to be the result of the subterranean saprophytic habit of the two generations. The possibility that the vascular strand in the gametophyte is vestigial, and thus of deeper significance, is mentioned here in view of the well-known hypothesis that the two generations in the Pteridophyte life-cycle are 'homologous', an hypothesis which has lately been adopted by Eames (1936) in his discussion of the Psilotaceae and other Lower Vascular Plants.

In their embryogeny *Psilotum* and *Tmesipteris* correspond closely. The

outstanding embryo features, now known to be shared by the two genera, features which, moreover, are not found in any other Pteridophyte, are the total absence of root and cotyledon, the strongly haustorial nature of the foot, the discarding of the foot by the young sporophyte so that for a considerable time the latter consists of shoot only, and the indefinite manner in which the apices of the shoot arise.

Here also it is possible to regard some or other of these peculiarities as the result of the saprophytic habit. It would, however, be highly improbable that such a group of specialized characters had arisen independently in the two genera. Thus the new facts brought forward with respect to the embryo of *Psilotum* may at least be held to push back the supposed specialization to a more or less ancient ancestral stock. That this stock is not a recent one is indicated by the considerable difference in sporophytic characters between the two existing genera. Moreover, the absence of even a rudiment of a root or cotyledon may further be considered to indicate that such specialization had been operating in this ancestral stock for a very considerable period.

In his latest publication dealing with the Psilotaceae Bower (1935) has elaborated the view that the lack of root and cotyledon has persisted in this group from a primitive stock which itself had never attained these organs. This view is strengthened by two quite distinct sets of facts, viz. those concerning the organization of the mature sporophyte of the Psilotaceae, and secondly those provided by the Devonian Psilotytales. It is not necessary to detail these facts here. That these Devonian plants were themselves not merely a local and specialized group is indicated by Lang's discovery (1937) of plants of similar nature from still earlier rocks of England and Wales.

SUMMARY

1. The occurrence of the gametophyte of *Psilotum triquetrum* in the volcanic soil of Rangitoto Island, Auckland, New Zealand, is described.
2. The form and structure of the gametophyte and of its vegetative gemmae and buds, and the development and mature structure of the antheridium and archegonium, are described.
3. The presence in the largest gametophytes of a conducting strand having annular and scalariform tracheids and a limiting endodermis is reported, and a description is given of the apical origin of this strand, its discontinuous character, and the structure of its tissues.
4. A number of stages in the development of the embryo are described, from which it is clear that the *Psilotum* embryo corresponds in all structural details with that of *Tmesipteris*.
5. The further growth of the embryonic shoot from one or more apices is described, and also its detachment from the foot.
6. The similarities and differences are noted between young gametophytes of gemma origin, young rhizomes of gemma origin, and young sporeling rhizomes.

7. The significance of the facts brought forward in this paper are discussed under the following heads:

- (a) The essential similarity of the gametophytes of *Psilotum* and *Tmesipteris*.
- (b) The occurrence of a vascular conducting tract in the largest gametophytes of *Psilotum* may, from different points of view, be interpreted as due either to some abnormal nuclear condition in such gametophytes, or to physiological changes taking place in the gametophyte as it grows in size, or to the persistence in it of an archaic feature.
- (c) The similarity of the *Psilotum* embryo and sporeling rhizome to that of *Tmesipteris* makes it more probable that the absence of root and cotyledon is a more or less archaic feature.

During the course of this investigation the writer communicated some of his results to Professor W. H. Lang and to Professor F. O. Bower, to each of whom he is much indebted for generous encouragement and advice. He also desires to record his thanks to Dr. R. B. Dodds, Dean of the Dental Faculty, University of Otago, for the use of photomicrograph apparatus, and to Mr. J. T. Holloway, for valuable help in taking the photographs.

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EXPLANATION OF PLATES VIII AND IX

Illustrating Dr. J. E. Holloway's paper on 'The Gametophyte, Embryo, and Young Rhizome of *Psilotum triquetrum* Swartz'

(All the figures are from untouched photographs)

PLATE VIII

FIG. 1. A median longitudinal section of the forking apex of a large gametophyte, showing antheridia and archegonia, the forward end of the axial conducting strand, and the endophytic fungus in advance of the latter. $\times 32$.

FIG. 2. A median longitudinal section of a portion of a large gametophyte, showing the axial conducting strand, surrounded by the starch zone, and antheridia at the surface. Tracheids are present in the strand. $\times 50$.

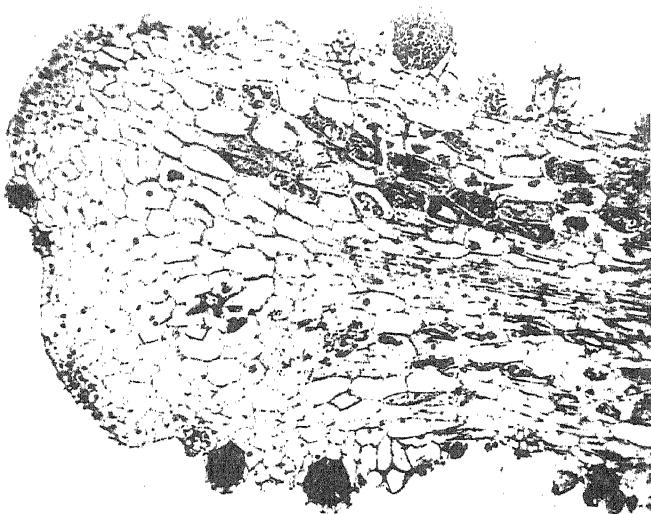
FIG. 3. Part of a transverse section through a large gametophyte, showing the axial conducting strand in which are three thick-walled tracheids, and an antheridium and an archegonium at the surface. $\times 60$.

FIG. 4. The axial tissues of the same section illustrated in Fig. 3, the three tracheids (which have not been touched) are clearly seen. $\times 200$.

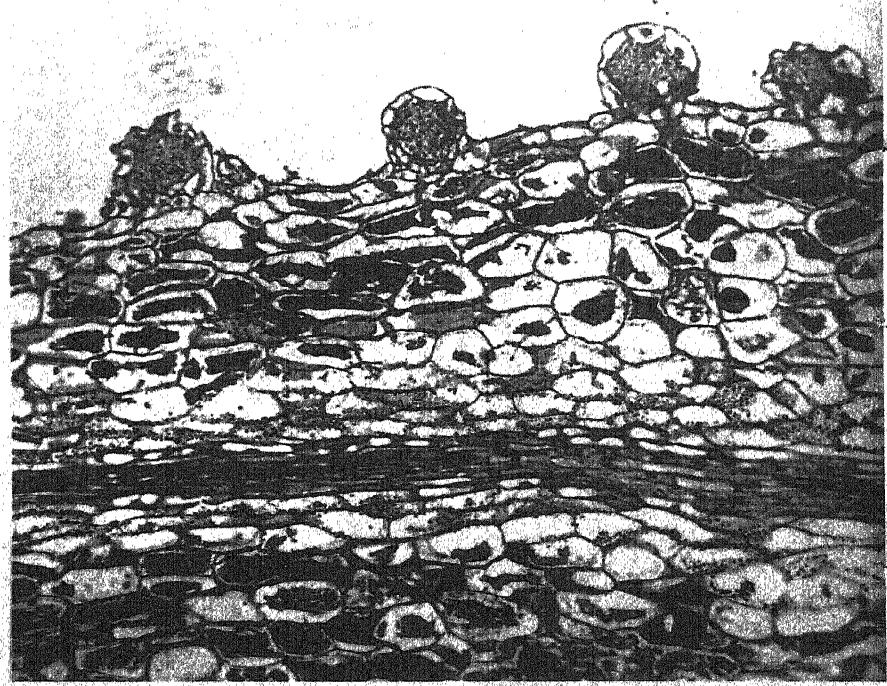
PLATE IX

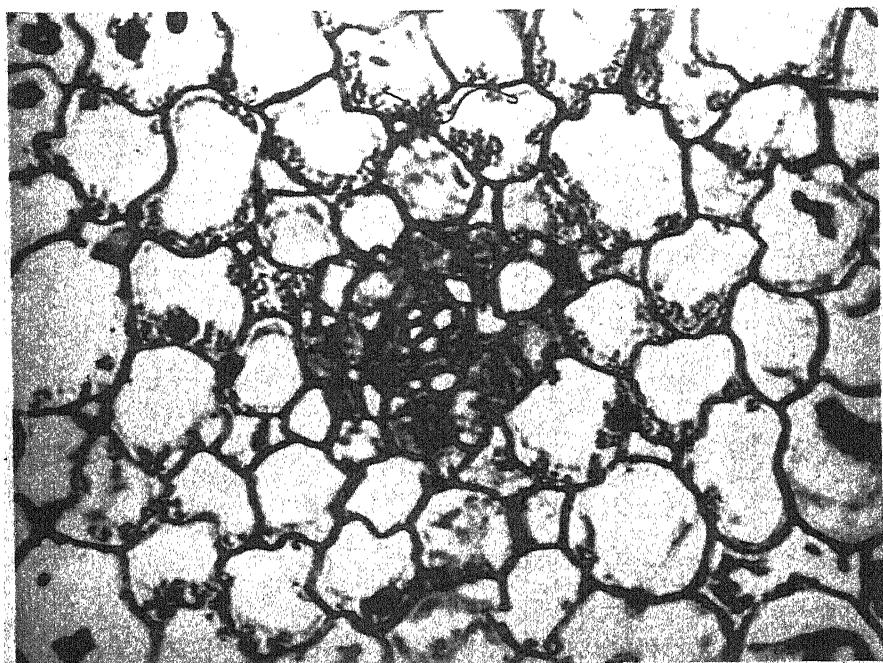
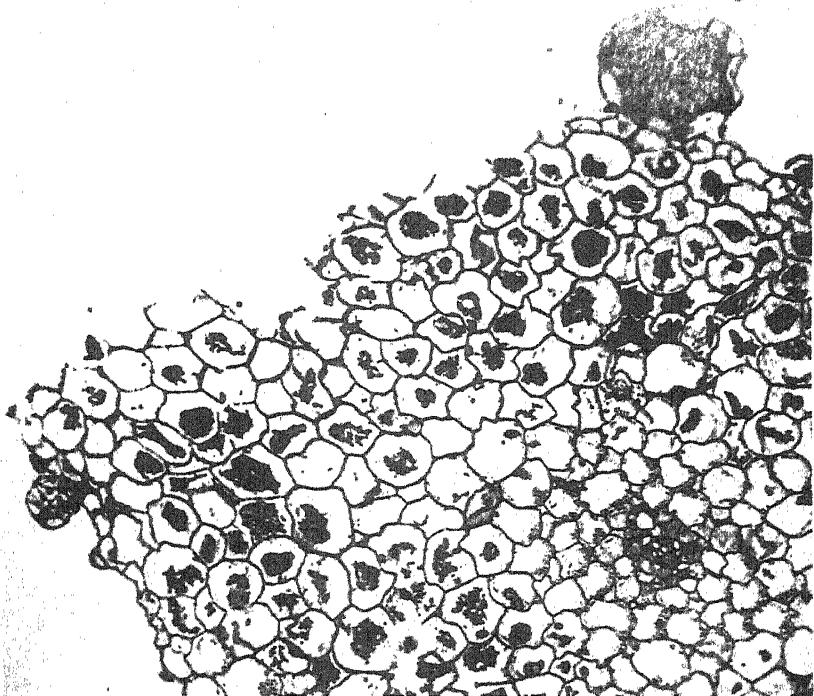
FIG. 5. A portion of another large gametophyte in median longitudinal section, showing tracheids in the axial strand, and an antheridium at the surface. $\times 95$.

FIG. 6. The axial tissues of the same section illustrated in Fig. 5, showing details of the tracheids. $\times 160$.



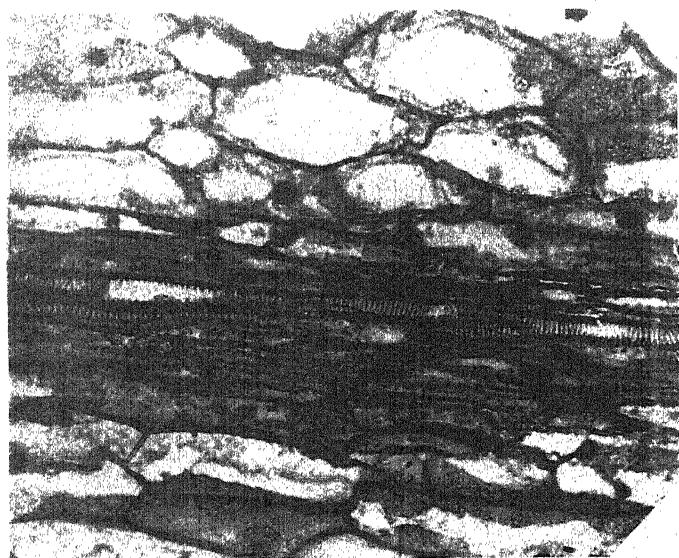
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Huth Stubb X. Kent.

HOLLOWAY — PSILOTUM.

Studies in Flower Structure

V. On the Interpretation of the Petal and 'Corona' in *Lychnis*

BY

AGNES ARBER

With five Figures in the Text

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I. INTRODUCTION

THE work detailed in the present paper was undertaken with two objects. The first of these was to try to throw light upon the nature of the outgrowths borne by the petals of *Lychnis*. The second was to see whether the structure of the flower in this genus could be regarded as compatible with the theory recently enunciated by Mattfeld (1938, 1938a), according to which the petal of the Caryophyllaceae is a duplex member, representing paired stipular appendages belonging to the stamen with which it is associated. In the following pages these two problems will be considered separately.

II. THE 'CORONA' OF LYCHNIS

(i) *Observations*

The male flower of *Lychnis vespertina* Sibth. may be taken as a convenient type in which to study the corolline outgrowths. Fig. 1A, p. 338, shows one of the petals with four outgrowths at the junction of claw and limb—two very delicate lateral wings, with two less fragile coronal teeth between them. In material collected near Cambridge, the coronal teeth have been found to be better developed in the male flower; in the female they tend to be rudimentary (Fig. 1, B). The corolla structure is variable, however, and suggests that the species might repay a study dealing analytically with the distribution and relation of the sexes, and with the characters—sex-linked or not—which distinguish the different forms.

Fig. 1, A, is drawn from a fresh petal, but C and D from petals stained whole to show the general vascular system of claw and limb. There are a median bundle and two laterals in the claw, while in the limb a network is produced by the branching of these three main strands. The median bundle bifurcates below the indentation of the limb. Preparations of the whole petal, such as

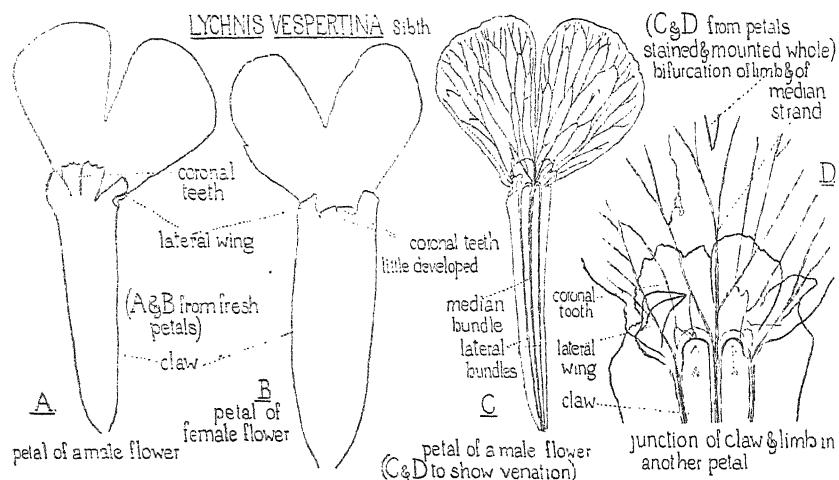


FIG. 1. *Lychnis vespertina* Sibth. A, petal of a male flower ($\times 2\frac{1}{2}$). B, petal from a bud (female) on the point of unfolding ($\times 2\frac{1}{2}$). C, petal of a male flower stained whole in gentian violet and eosin, and viewed from the front ($\times 2\frac{1}{2}$). D, junction of limb and claw from another petal treated as in C ($\times 9$ circa). This treatment shrinks the lateral wings and coronal teeth and renders them transparent; they can be better seen in A and B, drawn from fresh petals. The dotted arrows in D mark the mouths of the invaginations forming the coronal teeth.

those drawn in C and D, are not suitable for detailed examination of the outgrowths; for this purpose recourse must be had to transverse microtome series, such as those illustrated in Figs. 2, p. 339, and 3, p. 340. The junction of claw and limb in a single petal can be studied in Fig. 2, A1–A8, while further details of the formation of the corona are shown in Fig. 3. The delicate non-vascular wings are in process of detachment in Fig. 2, A2 and A3, and are completely free in A4–A8. In outline drawings of sections, such as those in Figs. 2 and 3, the wings are liable to look more substantial than they are in the living state, since the delicate mesophyll tears easily, and the upper and lower epidermal layers thus become too widely separated from one another.

At and above the level of detachment of the wings, two longitudinal wrinkles are formed in the petal limb, one on either side of the midrib, with a corresponding groove below each (Fig. 2, A3). On the upper surface the two wrinkles are separated by a narrow median groove, immediately above the midrib. If this midrib groove is followed downwards, its faces are found to fuse so as to leave an internal cavity (Fig. 2, A2, and B). The adjacent faces of the lateral grooves also fuse with one another at a level a little above their

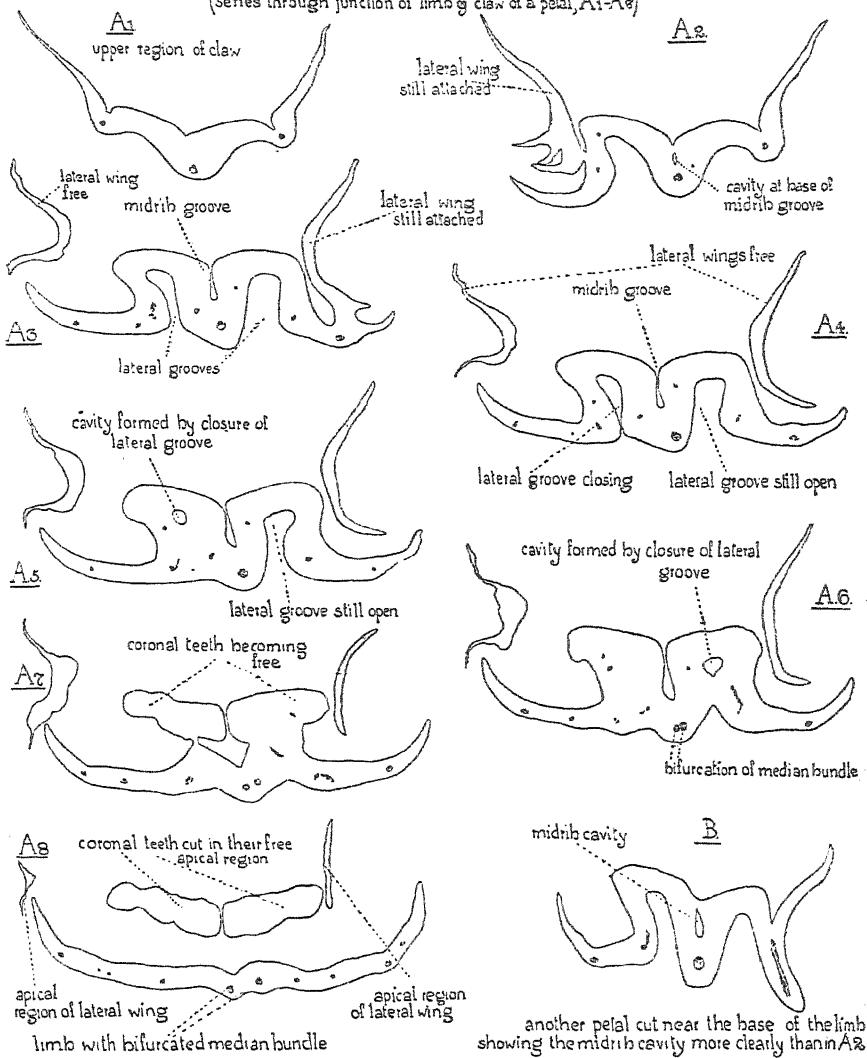
LYCHNIS VESPERTINA Sibth.(series through junction of limb & claw of a petal, A₁-A₈)

FIG. 2. *Lychnis vespertina* Sibth. A₁-A₈, sections from a transverse series from below upwards through the junction of limb and claw of a petal of a male flower ($\times 23$). B, transverse section of another petal from the same flower as the A series, cut near the base of the limb. ($\times 23$).

origin, leaving a small cavity within; this fusion has occurred on the left-hand side, but not on the right-hand side, in Fig. 2, A₅. The crown of each wrinkle, with its hollow centre, is prolonged upwards to form a free coronal tooth (Fig. 3, c₃ and c₄, p. 342). We may thus say that at the junction of claw and

LYCHNIS VESPERTINA Sibth.

(series through the junction of limb & claw of a petal, to supplement the series in preceding figure)

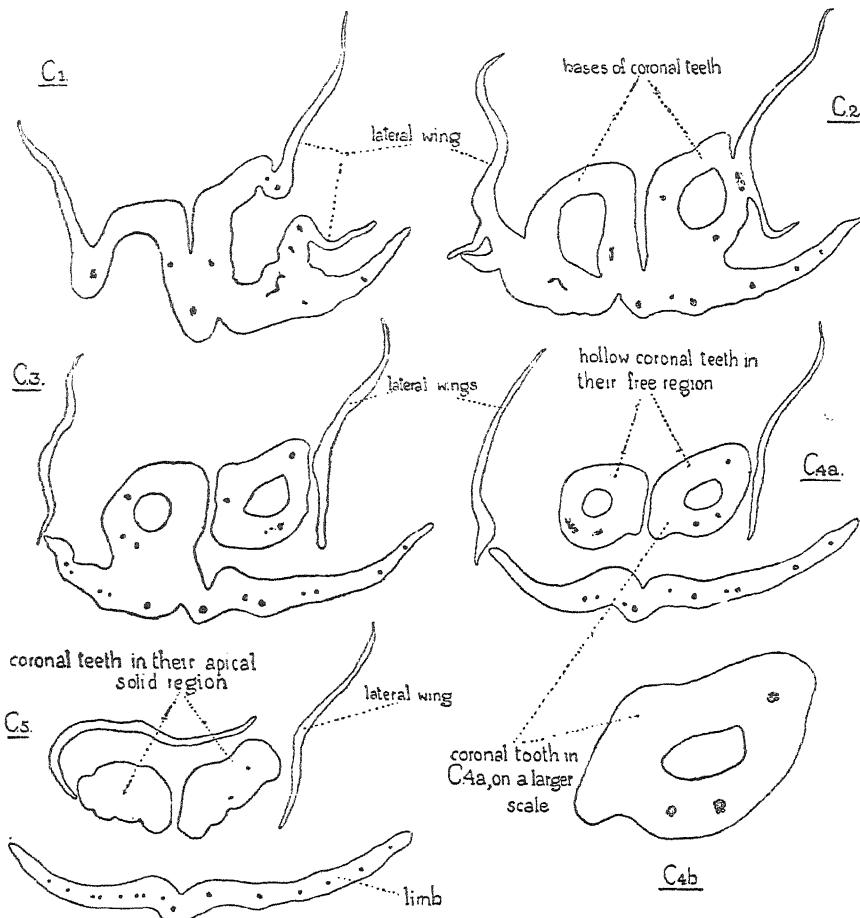


FIG. 3. *Lychnis vespertina* Sibth. c1-c5, sections from a transverse series from below upwards through a petal of a male flower ($\times 23$, except c4b); slightly damaged on the left-hand side of the petal, and reconstructed. c4b, shows one of the coronal teeth in c4a ($\times 47$); the xylem elements are indicated individually. (In this petal the attachment of the left-hand lateral wing is peculiar; the series cannot be followed below c1.)

limb there are three invaginations: the smallest of these is over the midrib, and opens in the upward direction on to the ventral surface of the petal, while the other two are lateral, and open in the downward direction on to the dorsal surface of the petal, in the positions marked with arrows in Fig. 1, D, p. 338. These lateral invaginations are continued upwards as free teeth projecting from the inner face of the petal, hollow below (Fig. 3, c4a), but solid at the

apex (Fig. 2, A8, and 3, c5). The teeth are only slightly supplied with vascular tissue, and such bundles as enter them die out before their tips are reached (cf. Fig. 2, A7 and A8). The external surface of each tooth belongs entirely to the upper petal surface, and the xylems of the bundles are all, as would be expected, directed towards this face (Fig. 3, c4b).

The construction of the coronal teeth is perhaps not easy to visualize from a description of sections; it may make it clearer if we imagine an enlarged version of the petal made of some extensible material which can be moulded at will, and suppose that two fingers are pressed into the back of the petal at the base of the limb—one on either side of the midrib—so as to force the petal into two glove-finger-like protrusions. These would represent the coronal teeth.

Lychnis vespertina is the only species of which I have cut microtome series through the coronal teeth. I have, however, cut hand sections of the petals of *L. diurna* Sibth. (male form) and *Silene maritima* With., and I have found that in both these species the coronal outgrowths conform to the same type as those of *L. vespertina*.

(ii) Discussion

The two kinds of outgrowth at the junction of claw and limb in *Lychnis*—‘lateral wings’ and ‘coronal teeth’—must be distinguished sharply from one another (cf. Velenovský, 1910); their structure, as shown in Figs. 2 and 3, is wholly different. The delicate, non-vascular lateral wings may be interpreted without special difficulty, for there seems no reason against comparing them with stipules. Glück (1919, pp. 92, 327) has pointed out that certain stipules of foliage leaves may be without vascular bundles, and illustrations of the non-vascular stipules of *Nasturtium officinale* R. Br. will be found in Arber, 1931, Fig. 8, p. 186.

The coronal teeth, on the other hand, are more problematic. They have generally been classed as ligular (e.g. Eichler, 1878; Pax, 1889; Glück, 1919; Rendle, 1925), and it has also been suggested that they may possibly prove to be stipular (Woodson and Moore, 1938). Velenovský (1910), on the other hand, regarded them as outgrowths whose orientation was opposite to that of the petals from which they arose; they would thus be comparable with the enation corona of *Narcissus* (cf. Arber, 1937). These three theories are all based on the supposition that the coronal tooth is a bifacial member, with an upper and a lower surface; if it is ligular or stipular, it should have its dorsal surface turned towards the ventral (upper) surface of the petal, and, if it is an enation, it should have its ventral surface turned towards the ventral surface of the petal. The observations recorded in the present paper demonstrate, however, that the tooth is not bifacial at all, since its free surface belongs exclusively to the *upper* surface of the petal; it represents a dorsal invagination of the petal, and can thus be neither ligular, stipular, nor an enation. These three possibilities being ruled out, the question arises as to whether there is

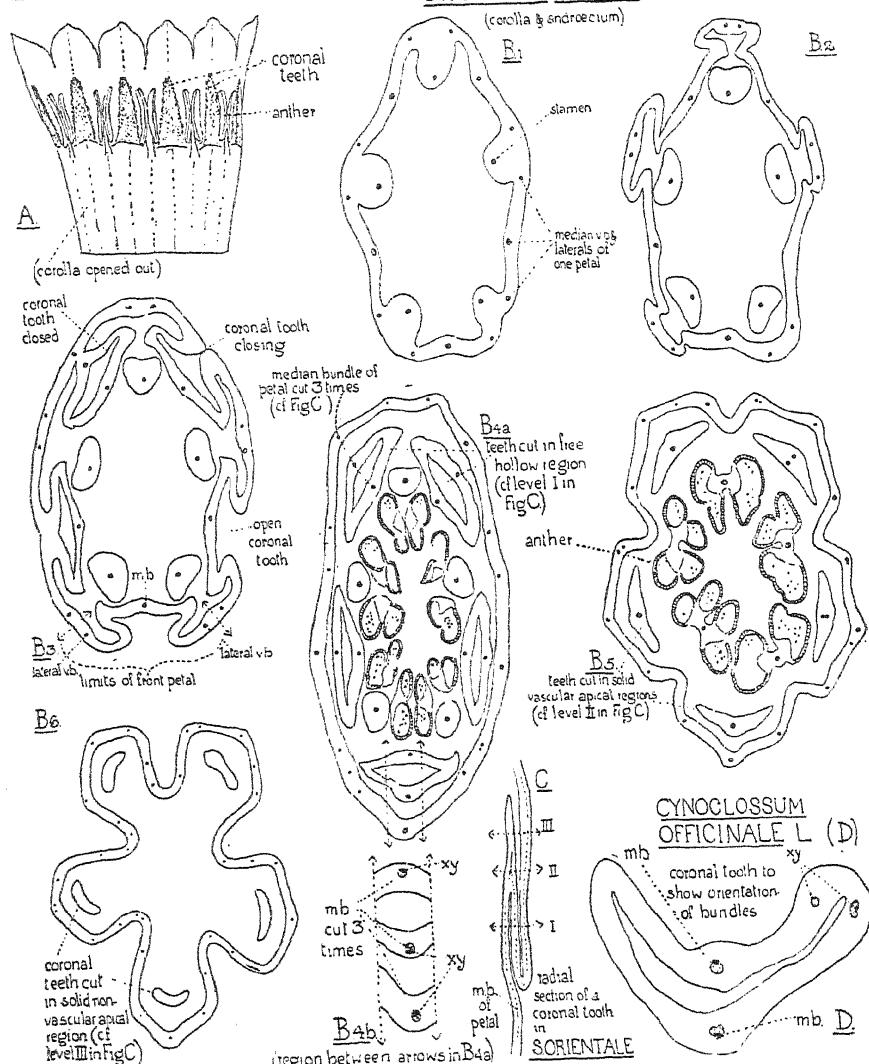
Coronal outgrowths in Boraginaceae

FIG. 4. Coronal teeth of Boraginaceae for comparison with Lychnis. A and B, *Sympodium aspernum* Donn. A, corolla opened out ($\times 2\frac{1}{2}$ circa), showing the coronal teeth with their papillose margins, opposite the petals; they are dotted for distinctness. B1-B6, sections from a transverse series from below upwards through a young flower ($\times 14$, except B4b); only corolla and androecium are included. B4b, sketch on a larger scale of the region between the arrows in B4a, to show the orientation of the median bundle, which is cut three times. C, *Sympodium orientale* L., radial longitudinal section of a coronal tooth from a series through a corolla ($\times 14$); the centre of the flower would be to the left. The median bundle of the petal is dotted. D, *Cynoglossum officinale* L., transverse section of a coronal tooth cut in its free region, to show the orientation of the vascular bundles ($\times 47$); the centre of the flower would be to the north.

any descriptive category to which these coronal teeth can be assigned. The closest parallel which I have been able to find for them is with the teeth (Hohlschuppen) in the mouth of the corolla tube of the Borraginoideae (cf. Gürke, 1897, and Brand, 1921, 1931). For comparison with Lychnis I have cut microtome series through the corollas of *Caccinia glauca* Savi, *Cynoglossum officinale* L., *Symphytum aspernum* Donn, *S. orientale* L., *Pulmonaria officinalis* L., and *Myosotis palustris* Lam. The construction of the corona in all these species is essentially similar; like those of Lychnis, the teeth are hollow invaginations (Einstülpungen) of the petal from the lower surface, ending in a solid apex. Their external appearance is shown in Fig. 4, A, p. 342; their structure will be understood from B1–B6, which represent transverse sections from a series through a corolla, passing from below upwards from the region in which the filaments are fused with the tube, to the tips of the coronal teeth. The teeth differ, however, in certain important respects from those of Lychnis. Each petal, instead of bearing *two lateral teeth* with little vascular tissue (as in Lychnis), bears *one median tooth* supplied by the median bundle itself, which passes up from the corolla tube within the inner wall of the tooth to its apex, then runs down within the outer wall, and then up again into the free part of the petal. This upward, downward, and upward course of the median bundle can be followed in transverse sections in Fig. 4, B1–B6, and in longitudinal section in c.

This comparison between the coronal teeth of members of the Silenoideae and Borraginoideae is made without any idea of suggesting a taxonomic relationship between plants belonging to widely different cycles of affinity. Types of structure, as such, may stand in a close morphological relation, which is wholly independent of phylogeny.

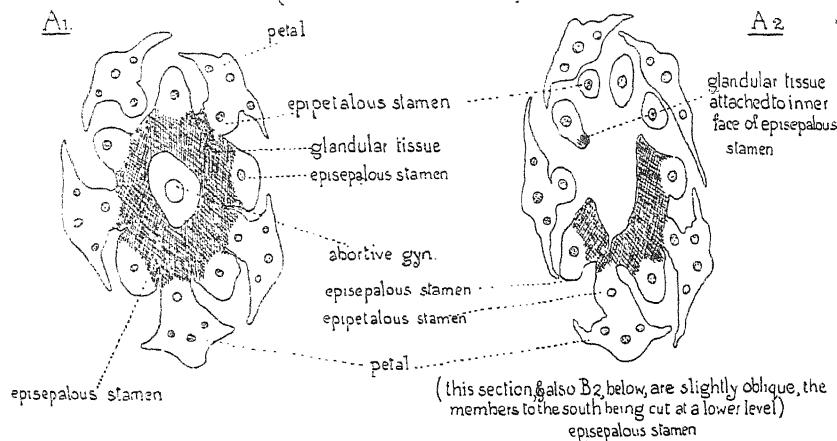
III. THE NATURE OF THE PETAL IN THE CARYOPHYLLACEAE, AND THE MEANING OF OBDIPLOSTEMONY

The connexion between the petal and its superposed stamen, in the Caryophyllaceae, is a striking feature to which attention has often been drawn in the literature. Recently a theory of the nature of the corolla in the Caryophyllaceae and other families—based upon the intimacy of the petal-stamen connexion, and upon the relation of the episepalous stamens to the nectary tissue—has been suggested by Mattfeld (1938, 1938a). This author regards the glandular tissue as staminal, and as representing stipule-like basal parts of the episepalous stamens. He considers that the petals are homologues of the glands; that is to say, he regards the petals as dorsal, stipule-like basal parts of the alternisepalous stamens. Thus an episepalous stamen, together with its gland, and an alternisepalous stamen, together with its petal, each consist of one phyllome only, and are homologous. This leads to the conclusion that the Caryophyllaceae have only two whorls between the sepals and the carpels. Mattfeld finds his theory on the Alsinoideae, in which the glands are particularly well developed, but since he proceeds to apply it to the

RELATION OF GLANDULAR TISSUE CORolla, AND ANDROECIUM IN LYCHNIS

LYCHNIS VESPERTINA Sibth (A)

(MALE FLOWER; CALYX OMITTED)



LYCHNIS ALPINA L (B)

(CALYX OMITTED)

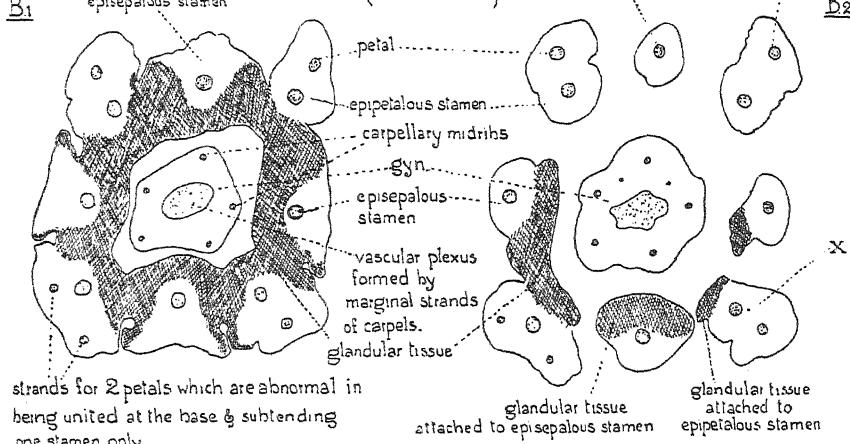


FIG. 5. A₁ and A₂, *Lychnis vespertina* Sibth., sections ($\times 23$) from a transverse series from below upwards through a young male flower, passing through the bases of the petals and stamens. B₁ and B₂, *Lychnis alpina* L. (garden material), sections ($\times 47$) passing through the bases of the petals and stamens from a transverse series from below upwards through a young flower; two sections were used in B₂. Throughout this figure the glandular tissue is shaded.

Caryophyllaceae as a whole, it may fairly be tested by considering it in relation to *Lychnis*, a member of the Silenoideae.

In Fig. 5, A₁, and also in A₂, which is cut at a slightly higher level, the bases of the stamens and petals of *Lychnis vespertina* are shown; the

glandular tissue is shaded. The basal attachment of the epipetalous stamens to the corresponding petals can be seen in both figures. On Mattfeld's theory we should expect to find the glandular tissue dorsal to the episepalous stamens, as the petals are dorsal to the epipetalous stamens; but the glandular investment is seen, on the contrary, to lie on the *adaxial* faces of the stamen bases, while the petal tissue is *abaxial*. This difference in their topographical relations makes it highly improbable that petals and glands are equivalent out-growths from the stamens. Another fact which is also irreconcilable with Mattfeld's theory is indicated in Fig. 5, B₂, drawn from *Lychnis alpina* L. It will be seen that, although the main part of the nectary tissue is attached to the bases of the episepalous stamens, a trace of it is carried up upon the bases of the epipetalous stamens to above the level at which the conjunct bases of petal and stamen leave the axis (e.g. the petal-stamen base marked X). The glandular tissue is thus not associated exclusively with the episepalous stamens, as it should be on Mattfeld's theory.

An unusual occurrence illustrated in the lower left-hand corner of Fig. 5, B₁ and B₂, is perhaps worth considering. The flower sectioned was abnormal in the fact that two of the petals were fused basally, though free at a higher level; their united bases were found to subtend *one stamen only*. This would be an unlikely contingency if the petals were stipules of the stamens, since it would mean that a single stamen could possess two pairs of stipules.

Apart from these detailed criticisms, a more general objection to Mattfeld's view is that it involves the supposition that two stipules belonging to the stamen have fused *abaxially*, i.e. behind the stamen. This author states (1938a p. 93) that such dorsal fusion may occur in the stipules of foliage leaves, and refers to the illustrations of *Althaea rosea* and *Hydrocotyle americana*, in Glück's book on stipules (1919), as examples in which 'die Nebenblätter der Laubblätter auf der Rückseite der Blattstiele verwachsen'. I cannot, however, detect, either in Glück's Fig. 68, p. 172 (*Althaea rosea* Cav.) or in Fig. 79, p. 188 (*Hydrocotyle americana* L.), any indication of the dorsal fusion of stipules which Mattfeld mentions, nor can I find any reference to it in Glück's text. It seems, indeed, to be a general rule that stipular fusion, if it occurs at all, occurs in front of the rest of the leaf, and thus any interpretation must lose probability if it postulates the dorsal fusion of stipules.

Mattfeld's theory, that the petal and its associated stamen form one phylome, has the apparent advantage that, by reducing the number of whorls recognized in the corolla and androecium, it may account for obdiplostemony in certain cases. If, however, his theory is not accepted, we can think of this obdiplostemony in still simpler terms by adopting the view that alternation of whorls is a matter of spatial and mechanical possibilities, rather than of morphological significance. In a study of the Fumarioideae (1931a) I pointed out that the lateral stamens belong *anatomically* to the inner whorl of the androecium, but *in the alternation sequence* they count as one whorl with the lateral petals on which they are superposed. This conclusion can be extended, with

modifications, to other families. In the Caryophyllaceae, for instance, the petal-stamen pair may behave in the alternation sequence either as a single member, or as two (if we make the natural assumption that only one whorl of carpels is represented throughout the family); for certain genera have epipetalous carpels, i.e. *petal+superposed stamen* count as two separate members, while others have epipetalous carpels, i.e. *petal+superposed stamen* counts as one member. This facultative behaviour must be held to show that two members may form a unit in whorl alternation without being a unit in the morphological sense; unless we accept this conclusion, we shall be in the untenable position of interpreting the petals differently in different genera of the same family. On the view that whorl alternation may be independent of the morphological status of the members concerned, obdiplostemony becomes a problem of shoot-apex mechanics, rather than of comparative morphology.

IV. SUMMARY

1. It is shown that the two coronal teeth of the petal in *Lychnis* are not ligular or stipular, neither are they enations; but they are hollow lateral invaginations, corresponding in general structural type to the single median tooth in the Borraginoideae.
2. Evidence is tendered that is held to disprove Mattfeld's theory (1938, 1938a) that the petal in the Caryophyllaceae represents fused stipular out-growths from the superposed stamen.
3. The suggestion is made that the spatial relation of floral whorls is due to mechanical causes, and hence that the 'problem' of obdiplostemony is, for the morphologist, an imaginary one.

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An Investigation of *Plumaria elegans* (Bonnem.) Schmitz with Special Reference to Triploid Plants bearing Para- sporangia

BY

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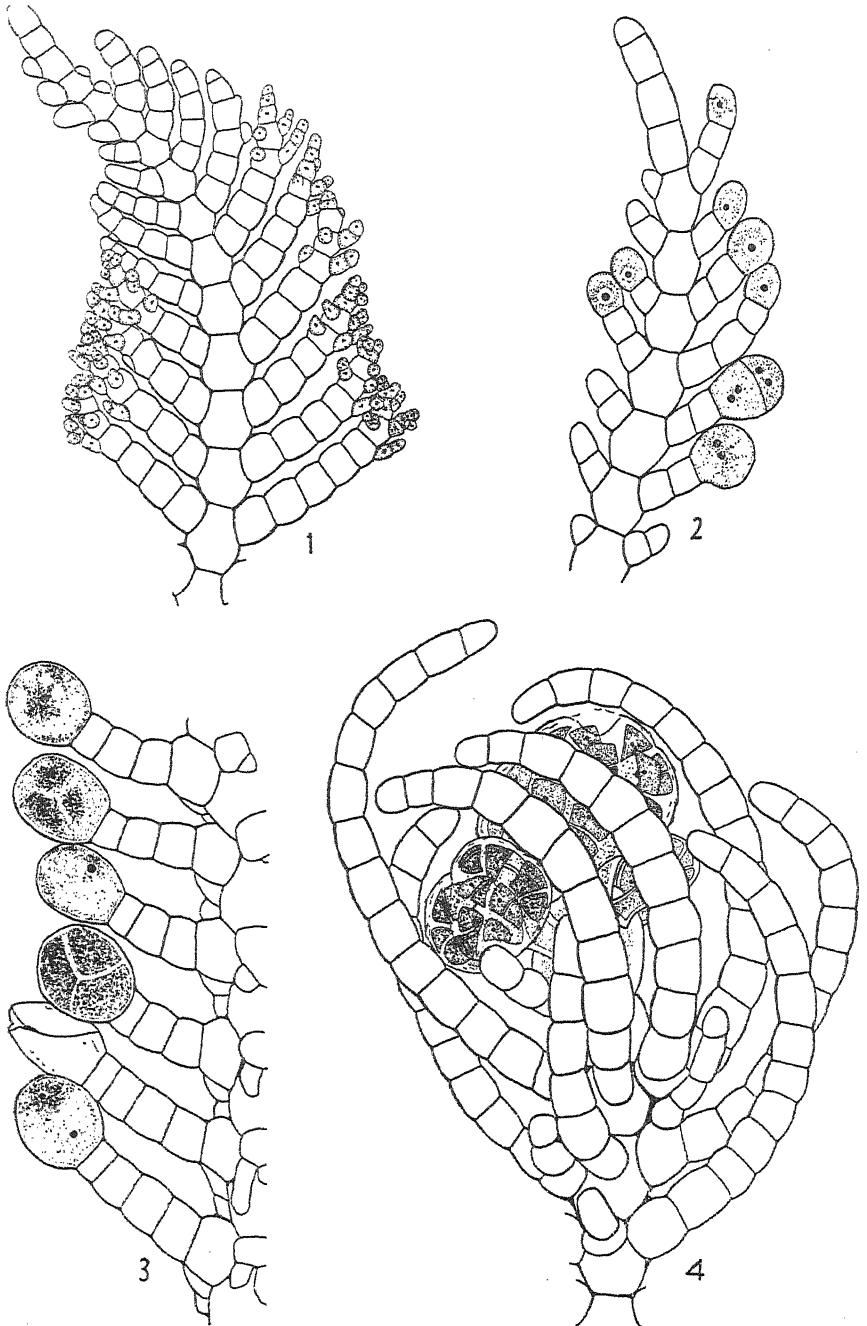
With Plate X and thirty-five Figures in the Text

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I. INTRODUCTION

IN an earlier paper (Drew, 1937) the nuclear history of the polysporangium of *Spermothamnion Snyderae* Farlow was described. This polysporangium was shown to be homologous with a tetrasporangium both in origin and function, the only difference being in the greater number of spores formed. This was found to be due to the fact that the polysporangium initial contains several nuclei instead of one. All the nuclei undergo reduction division and hence several tetrads of spores result. That investigation has led on to the present study of the cytology of another type of sporangium found in the Ceramiales, the parasporangium. Like the polysporangium, it contains several spores. *Plumaria elegans* (Bonnem.) Schmitz seemed an obvious choice for such an investigation as it is a readily available plant, being one of the commonest algae on the coasts of the British Isles and the parasporangia are produced in abundance. It is also known from other European countries including Spain, France, Denmark, Sweden, South Norway, the Faroes, and Iceland, and in addition from the American shores of the North Atlantic.

P. elegans belongs to the Ceramiaceae. The thallus is filamentous, the branching very regular and in one plane. Growth is brought about by divisions of the apical cells, and at a very short distance behind the apex cortication is initiated and develops extensively over the main axes. The lower orders of branchlets remain uncorticated. In addition to the plants which bear parasporangia, there are some which bear tetrasporangia and yet



TEXT-FIGS. 1-4. Fig. 1. Apical portion of a branch showing early stages in development of spermatangial branchlets. Fig. 2. Branchlet bearing young parasporangia. Fig. 3. Tetrasporangia in various stages of development. Fig. 4. Cystocarp with two mature gonimolobes and others in the process of development. All $\times 275$.

others which bear sexual organs. As the spermatangia and carpogonia occur on separate individuals this means that there are four types of plants, distinguishable by the reproductive organs borne (Text-figs. 1-4). Usually the plants on which the parasporangia develop bear no other reproductive organs, but in some instances a few tetrasporangia are initiated, and a small percentage of these may even mature (Text-fig. 35). This is doubtless the explanation of the statements that parasporangia appear to develop on tetrasporic plants (Kylin, 1937) and that parasporangia are the result of further divisions in the tetrasporangia (Harvey, 1853; Hauck, 1885; Taylor, 1937), neither of which statements can be supported by the observations of the present writer. The parasporangia have also been confused with the cystocarps, but in reality they can be easily distinguished from one another. Whereas the parasporangium is naked (Text-fig. 33), the numerous gonimolobes of a single cystocarp are enveloped by several filaments (Text-fig. 4) and each gonimolobe itself contains many more spores than the largest parasporangium.

A study of the parasporangium and plants bearing them has led to the rather unexpected discovery that these are triploid relative to the sexual and tetrasporic plants which are haploid and diploid respectively. This, being so far unique in the algae, the main purpose of this paper is to present the evidence on which this conclusion is based. The investigation has naturally involved cytological study of all four types of plant and a critical inquiry into the normal life-cycle. This has in turn necessitated an investigation of the morphology of the reproductive organs, for while the vegetative structure has been known in detail for over seventy years (Cramer, 1864), existing descriptions of the sexual organs, especially the procarps, are incomplete. In presenting the morphological section of the paper, which for convenience is placed first, it is realized that there is unavoidable detail, due to the nature of the subject. Those readers not especially interested in those details will probably obtain sufficient information to enable them to follow the cytological findings by reference to Text-figs. 1-35.

2. MATERIAL AND METHODS

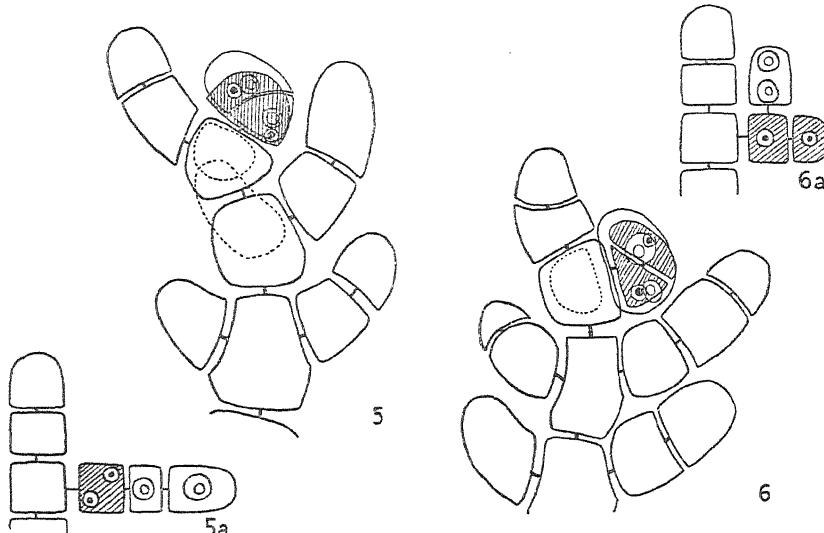
With the exception of a valuable collection from Kristineberg, Sweden, the plants used in this investigation have been collected on the coasts of England and Wales at various times of year.

P. elegans has proved a comparatively easy alga to handle. The material has been fixed, in most cases, immediately after being taken off the rocks where it was growing, in a mixture of 100 c.c. of 70 per cent. alcohol and 6 c.c. of 40 per cent. formaldehyde and kept in that fixative indefinitely. As soon as the plants were sufficiently bleached (a process hastened by changing the solution) the portions required for investigation were stained in Brazilin and mounted whole in Canada balsam, using the method described for *Spermothamnion Turneri* (Drew, 1934). The same optical equipment has been used as in that investigation.

3. THE DEVELOPMENT AND STRUCTURE OF THE REPRODUCTIVE ORGANS

A. Procarps and cystocarps.

The first description of the procarp of *P. elegans* was given by Bornet and Thuret (1876) and their observations although correct were incomplete. Later writers (Davis, 1896; Phillips, 1897; Kylin, 1923) have dealt in the main with



TEXT-FIGS. 5-6. Diagrammatic drawings of two young procarps from same branchlet showing irregularity of order of early divisions. Fig. 5. Supporting cell (shaded and with black nuclei) binucleate and carpogonial branch (unshaded and white nuclei) which is underneath supporting cell, two-celled. Fig. 6. Sterile cell cut off from supporting cell (both shaded and with black nuclei) underneath carpogonial branch initial (unshaded and white nuclei) which is undivided but binucleate. Both $\times 850$. Figs. 5a and 6a diagrams to show cell connexions of these procarps. Shaded in same way as drawings.

neighbouring species, but Phillips gives a diagrammatic drawing of the mature procarp of *P. elegans*.

The procarp arises near the apex of the lesser branchlets of limited growth. The sub-apical cell or one very near the apex cuts off, on its abaxial side, a lateral cell, *s*, which is remarkable on account of its large size and the size of the nucleus (Text-fig. 7). This is the supporting cell, and a sterile cell arises from one side of it and the initial of the carpogonial branch from the other. A study of the early stages has not provided conclusive proof as to which of these two cells is formed first, but there is evidence which suggests that the order in which the early divisions take place is not fixed rigidly. In the example figured in Text-fig. 8 the first-formed cell has the appearance and position of the sterile cell, but in another case seen, the first-formed is undoubtedly the carpogonial branch initial, as in *Ptilota plumosa* (Kylin, 1923). Two young procarps, developing near together on the same axis and figured diagram-

matically in Text-figs. 5 and 6, also illustrate the fact that the order of the early divisions is not fixed.

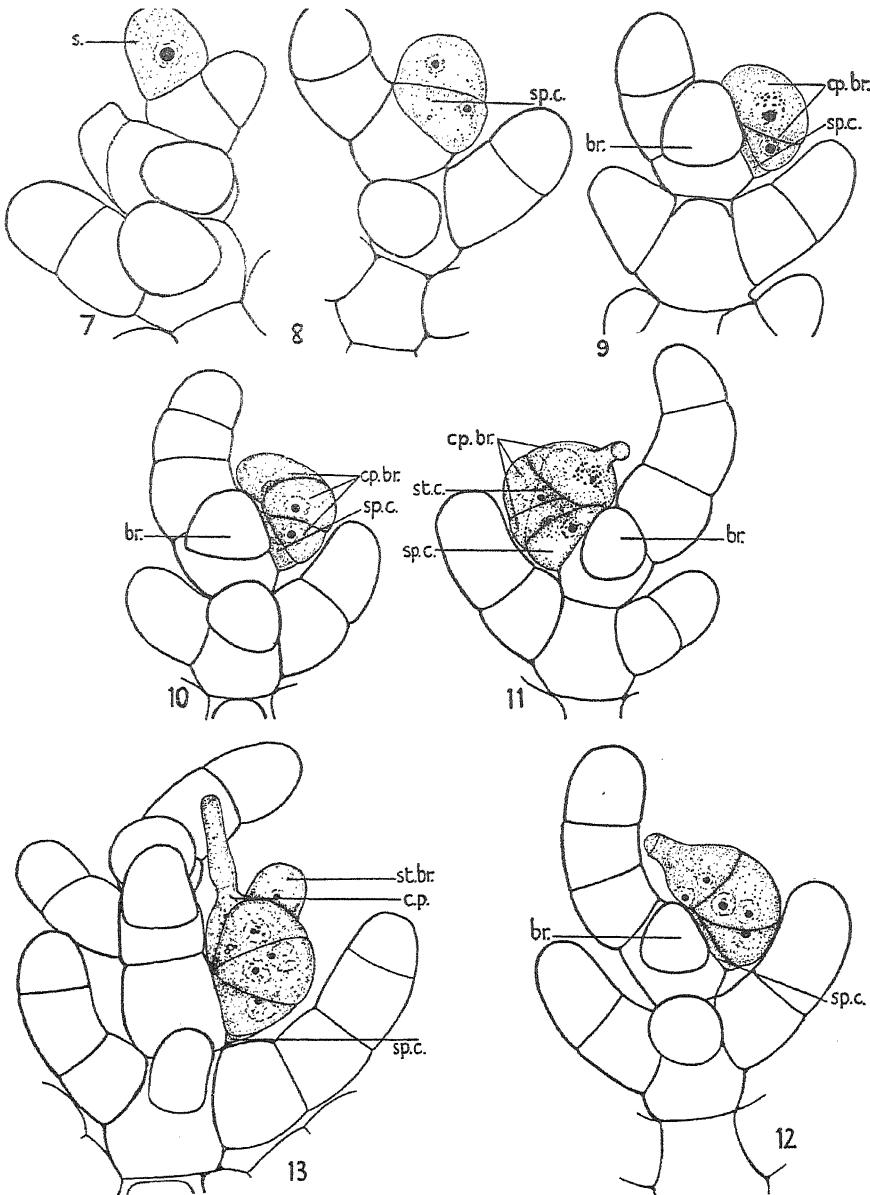
The sterile cell divides once or twice before fertilization to form a short branchlet (Text-figs. 18-22), and at the same time the carpogonial branch initial divides to give four cells (Text-figs. 9-13 and 19-22). These cells are wedge-shaped and curve round the supporting cell, the apical cell forming the carpogonium with a trichogyne. The carpogonium (Text-fig. 13, *c.p.*) is remarkably small and is usually hidden between the third cell of the carpogonial branch on one side and the sterile branchlet on the other. As will be seen in the figures referred to, the cells of the carpogonial branch are uninucleate, excepting the second cell, which is binucleate. No trichogyne nucleus has been seen.

Meanwhile, the apical cell of the main axis divides three or four times (Text-figs. 7-13 and 18-22). The resulting cells arch over the carpogonial branch, which superficially appears to be terminal on the axis in consequence. The axial cells in the vicinity of the carpogonium tend to branch profusely, the fertile segment usually giving rise to two branchlets (Text-figs. 9-12, *br.*), one on either side of the supporting cell. These branchlets are not confined to one plane as are those of the vegetative parts of the plant (Text-fig. 13).

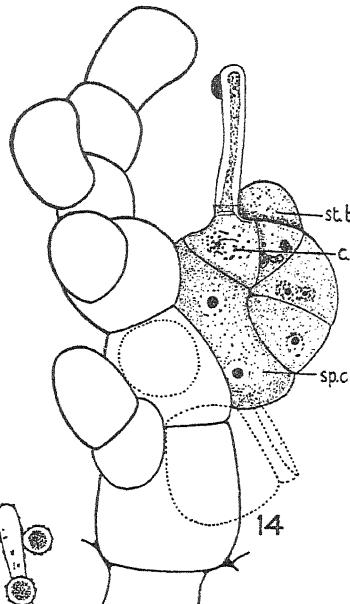
After fertilization, the protoplast of the carpogonium becomes separated from that of the trichogyne and the side of the cell against the supporting cell enlarges (Text-fig. 14, *c.p.*). The supporting cell itself grows very considerably and after a division of the nucleus (Text-fig. 14, *s.p.c.*) it divides into a smaller lower portion, known as the basal cell (*b.c.*), and a larger upper portion, the auxiliary cell (Text-fig. 15, *a.c.*).

The carpogonium nucleus stains very slightly both before and after fertilization, but whereas before fertilization it is small and there is a single very small nucleolus, afterwards it is considerably larger and there are two or three nucleoli. The fusion nucleus divides once in the carpogonium, and one of the resulting daughter nuclei is cut off with the corner of the carpogonium most in contact with the auxiliary cell, to form the sporogenous cell (Text-fig. 15, *s.c.*). After the formation of this cell, the rest of the carpogonial branch begins to degenerate and the nucleus of the auxiliary cell moves to the basal end of that cell (Text-fig. 15). It is still in that position when the diploid nucleus passes into the apical end after connexion is established between the sporogenous cell and the auxiliary cell. No indication of a fusion between these nuclei has been seen and they remain easily distinguishable, as the haploid nucleus is large with a prominent nucleolus and stains deeply while the diploid nucleus, derived from the fusion nucleus, has now no nucleolus and takes up very little stain.

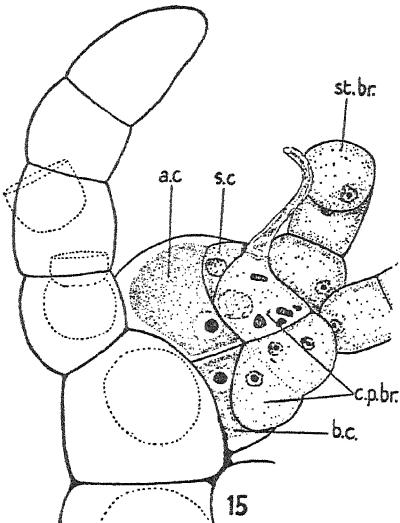
Very soon the auxiliary cell is divided by a wall parallel to that of the basal cell, into a very small lower portion, called the foot cell (*f.c.*), and a very large apical portion, the central cell (Text-fig. 16, *c.c.*). The foot cell eventually contains two or three nuclei derived from the haploid auxiliary cell nucleus



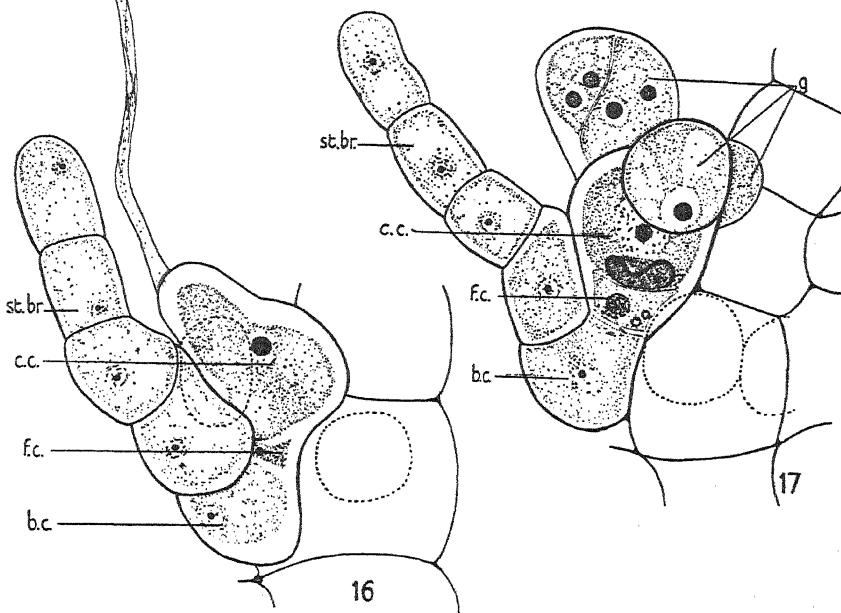
TEXT-FIGS. 7-13. The development of the carpogonial branch. $\times 850$. Fig. 7. The lateral segment (*s.*), on which the branch develops. Fig. 8. Division of segment. Lower cell is supporting cell (*sp. c.*) and upper probably sterile cell. Fig. 9. Carpogonial branch (*cp. br.*) two-celled and almost covering supporting cell (*sp. c.*). Nucleus of apical cell of carpogonial branch in prophase. Fig. 10. Carpogonial branch (*cp. br.*) three-celled. Fig. 11. Carpogonial branch (*cp. br.*) still three-celled but trichogyne has started to develop and nucleus of apical cell in prophase. Supporting cell (*sp. c.*) and sterile cell (*st. c.*) in front of carpogonial branch. Sterile cell indicated by broken line. Fig. 12. Slightly older carpogonial branch showing binucleate condition of both apical and subapical cells. Fig. 13. Mature four-celled carpogonial branch. Apical cell (*st. br.*) of sterile branchlet, which is behind carpogonial branchlet, just showing. (*c.p.* = carpogonium.)



14



15



16

17

TEXT-FIGS. 14-17. Post-fertilization developments. $\times 850$. Fig. 14. Fusion nucleus in carpogonium (*c.p.*) in prophase and supporting cell (*sp. c.*) which has enlarged considerably, binucleate. Fig. 15. Sporogenous cell (*s.c.*) formed and supporting cell has divided into auxiliary cell (*a.c.*) and basal cell (*b.c.*). Fig. 16. Foot cell (*f.c.*) formed and central cell (*c.c.*) protruding to form first gonimolobe initial. Remains of carpogonial branch of which trichogyne alone is visible, behind central cell. Fig. 17. One very young gonimolobe and two initials (*g.*) formed. Nucleus of central cell (*c.c.*) in prophase.

and it is not long before the whole cell degenerates. The central cell encloses the diploid nucleus only. After enlargement and the formation of a nucleolus, this nucleus divides and one of the daughter nuclei moves into an apical protuberance which is then cut off and forms the initial of a gonimolobe. This process is repeated two, three, or four times and often in quick succession (Text-fig. 17). The first wall laid down in a gonimolobe initial is always oblique, and finally a large mass of cells results from repeated divisions. Every cell of this mass, excepting the one in contact with the central cell, forms a carpospore. At maturity, the spores are deeply coloured and angular. The protoplasmic connexions then disappear and the spores become separate and round up somewhat before they are liberated by a rupture in the surrounding wall (Text-fig. 4).

Immediately after fertilization the branchlets in the vicinity of the procarp, including the branchlet formed from the sterile cell, quickly grow up around the developing gonimolobes (Text-fig. 4). Some of these terminate in hairs.

In figures such as Text-figs. 7-17 only the side of the fertile branchlet towards the observer can be shown, and hence a set of diagrams showing the complete structure is added (Text-figs. 18-27). The carpogonial branch is here shown in red on a transparent sheet. The diploid fusion nucleus is shown dotted in red also on this sheet, but it should be remembered that it moves from a cell shown in red outline to one with black outline.

B. Spermatangia.

The spermatangia were first described with any certainty by Buffham (1891). They are formed in great profusion and usually the greater part of a branch is concerned in their production. Text-fig. 1 shows the apical portion of a branch with spermatangial branchlets in early stages of development. It will be noticed that with the successive transverse divisions of the apical cell of a branchlet, there is a rapid diminution in the size of the cells, the final apical cell being very small. The last four or five cells branch profusely, not only in one plane as in the vegetative parts of the plant, but on all sides of the axis. On the resulting branchlets, which are very small, spermatangia develop either directly or after still further branching. Each mother cell gives rise to two or three spermatangia, which are minute subspherical cells, each liberating one spermatium.

C. Tetrasporangia.

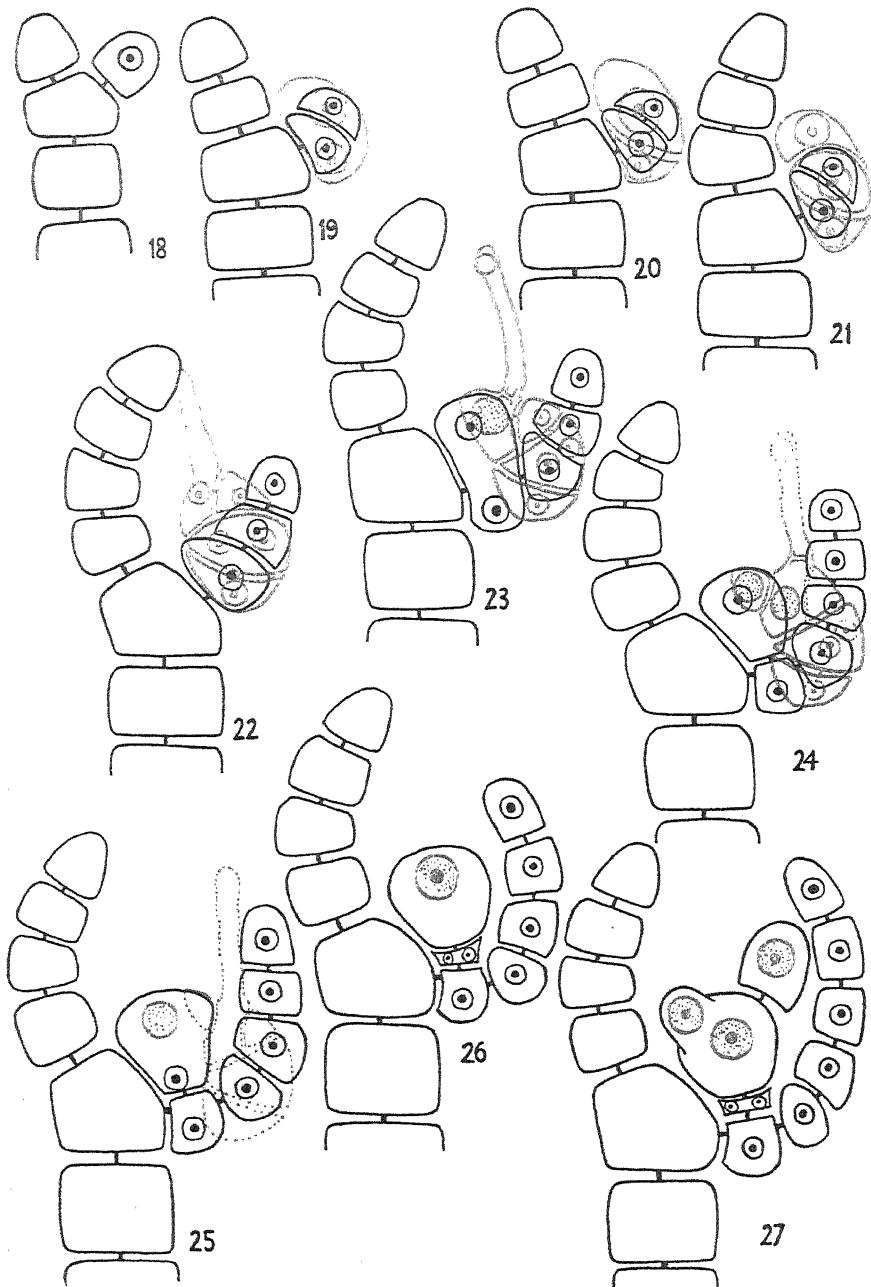
The tetrasporangia present no features calling for special remarks. Text-fig. 3 shows a group of sporangia in various stages of development. They are borne usually terminally, rarely laterally, on the lower orders of branchlets. The initial is uninucleate and the two nuclear divisions are completed before any division of the protoplast takes place. This is brought about by invagination and the four spores which result are tetrahedrally arranged.

D. Parasporangia.

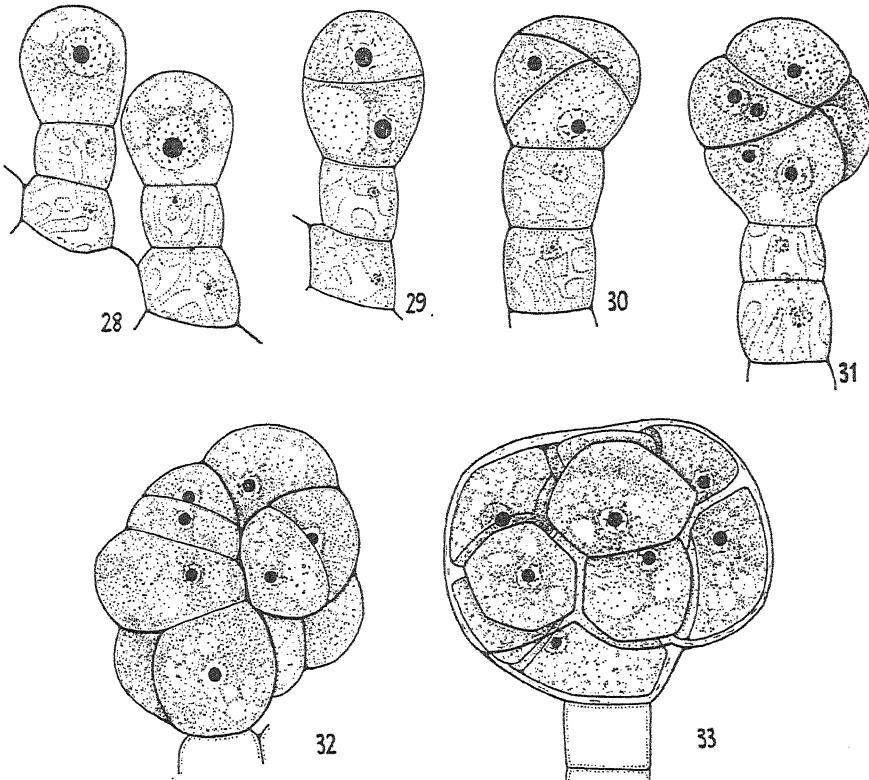
The parasporangia of *P. elegans* were first described by Pringsheim (1862) from material collected on Heligoland, and one of his figures is familiar by frequent reproduction. They were referred to by him as favellae on account of a supposed resemblance to gonimolobes, and they have sometimes been called naked favellae by other writers. Mature parasporangia are of irregular shape and varied sizes and they contain deeply coloured angular spores, usually numbering between ten and twenty-four (Text-fig. 33). They frequently occur in large numbers all over the plant, but sometimes develop in definite zones. The parasporangia occupy terminal positions on the lower orders of branchlets.

A parasporangium originates from a single cell (Text-figs. 2 and 28), which is at first indistinguishable from a tetrasporangium initial and occupies an identical position. The initial increases in size and rounds up so that it is almost spherical by the time of the first nuclear division. Following the division of the nucleus, the cell is divided by a wall, which may develop in any plane whatsoever. The daughter cells then divide and the process is repeated several times (Text-figs. 29-32). The cells may or may not divide at the same time. The latter condition is shown by the sporangium of Text-fig. 31, where the nuclei of two of the four cells have divided already and the nuclei of the remaining two are in prophase. The planes in which the new walls are laid down appear to be entirely fortuitous, as a glance at Text-figs. 29-33 will show. The irregularity increases with every division. After a while, cell division ceases and the protoplasm becomes denser (Text-fig. 32). Plastids, which have not been visible in the initial and during development, appear. Finally the protoplasmic connexions go and the walls between the cells swell up and disappear or liquefy, since the spores are liberated by the rupture of the external wall of the sporangium only. This wall has become thick and slightly lamellose by maturity (Text-fig. 33). Rosenvinge (1923-4) states that the spores are naked when liberated.

It is a frequent occurrence for the nuclei of young, partly developed sporangia to cease to divide and to pass into the quiescent condition characteristic of cells which have likewise ceased to develop and divide. The protoplasm of these cells becomes much less dense and pale plastids appear (Text-fig. 34). Sometimes only part of the parasporangium is thus affected. Such cells may remain in this condition permanently, or on the other hand one or more of the group may become active again and then each grows out into an uncorticated filament. Examples have been seen where other parasporangia have then developed from the apical cell of such filaments. In the case of the specimen figured no fewer than three cells of the original parasporangium have behaved in this way (Text-fig. 34). One of the new filaments is terminated by a parasporangium initial, *p. 1*, and another by a parasporangium consisting of several cells, *p. 2*.



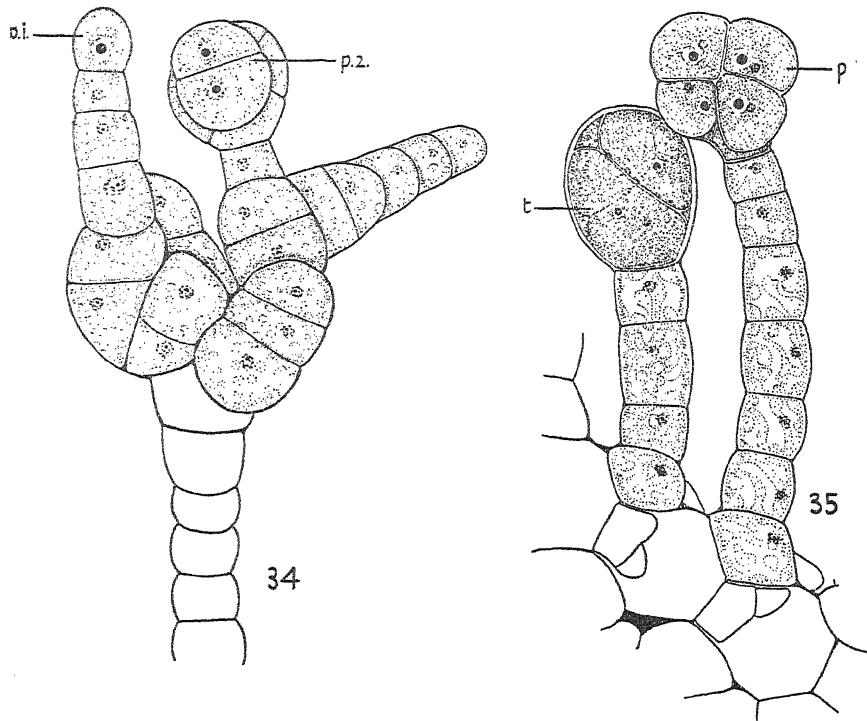
TEXT-FIGS. 18-27. Diagrams showing the development of the carpogonial branch (Figs. 18-22) and the origin of the cystocarp (Figs. 23-7). The carpogonial branch is shown in red and the other cells in black. The fusion nucleus and all nuclei derived from it are shown in red also, but the nuclear area is dotted.



TEXT-FIGS. 28-33. The development of the parasporangium. $\times 625$. Figs. 28-31. Very young stages. Fig. 32. Sporangium, in which divisions have probably ceased. Note the increasing density of the protoplasm and the decreasing size of the nuclei. Fig. 33. A mature parasporangium with the spores lying free, ready for liberation.

4. CYTOLOGICAL INVESTIGATION

The apical uncorticated regions of the branch systems of *P. elegans* provide excellent material for the study of vegetative nuclei in division. The cells are uninucleate and divide frequently in actively growing material. The nuclei are comparatively large so that in late prophase when the chromosomes are scattered in the nuclear area around the nucleolus, they can be counted with a good degree of accuracy. An examination of such nuclei shows that there are haploid, diploid, and triploid plants and examples of such nuclei are shown in Figs. 1-4 on Pl. X. Figs. 1 and 2 are nuclei in cells of a male and female plant respectively. Thirty-one chromosomes, the haploid number, are present in both of these nuclei, although one is hidden behind the nucleolus in Fig. 1 and two are similarly hidden in Fig. 2. The nucleus of Pl. X, Fig. 3, is from the apical cell of a plant bearing tetrasporangia and is diploid. Of the sixty-two chromosomes present in this nucleus two are absent in the figure, one



TEXT-FIGS. 34-5. Fig. 34. A degenerate parasporangium, cells of which have given rise to three filaments, one bearing a parasporangium initial (p. i) and another a several-celled parasporangium (p. 2). $\times 450$. Fig. 35. Portion of an axis bearing a parasporangium (p.) and a tetrasporangium (t.), on neighbouring branchlets. $\times 450$.

being behind another chromosome and the other behind the nucleolus. Pl. X, Fig. 4, is of a triploid nucleus from the cell of a plant bearing parasporangia. The triploid nuclei are much more difficult to count exactly, for although the nuclear area is usually slightly larger, the chromosomes are more crowded together. Thus in the case of the nucleus figured it is not possible to tell whether three, marked *a*, are single or double, and so the total is ninety or ninety-three. Five of these chromosomes are not figured as they are behind other chromosomes or the nucleolus. The nucleus of an apical cell of a branch of a parasporic plant from Sweden is also figured (Pl. X, Fig. 16). Ninety-two or ninety-three chromosomes are distinguishable in this nucleus, the structure marked *a*, being either one dividing chromosome or, more probably, two very close together. Seven of the total number of chromosomes do not show in the figure as they are under the nucleolus or other chromosomes. It will be noticed that this nucleus is smaller than that of the corresponding English material. This difference is constantly displayed in the material examined, but the reason for it is obscure.

The chromosomes of *P. elegans* differ from those of *Spermothamnion Turneri* and *Spermothamnion Snyderae* in having a greater variety of size and shape. Some are very long, others are two to three times their width and sausage-shaped, but the majority are spherical. Some of the latter are exceedingly small. It has not been possible to determine how many of each type are present, and to check their repetition in the diploid and triploid on account of their very small size and the fact that the appearance of a particular chromosome depends so much on its position. In the haploid set there are probably three very long ones.

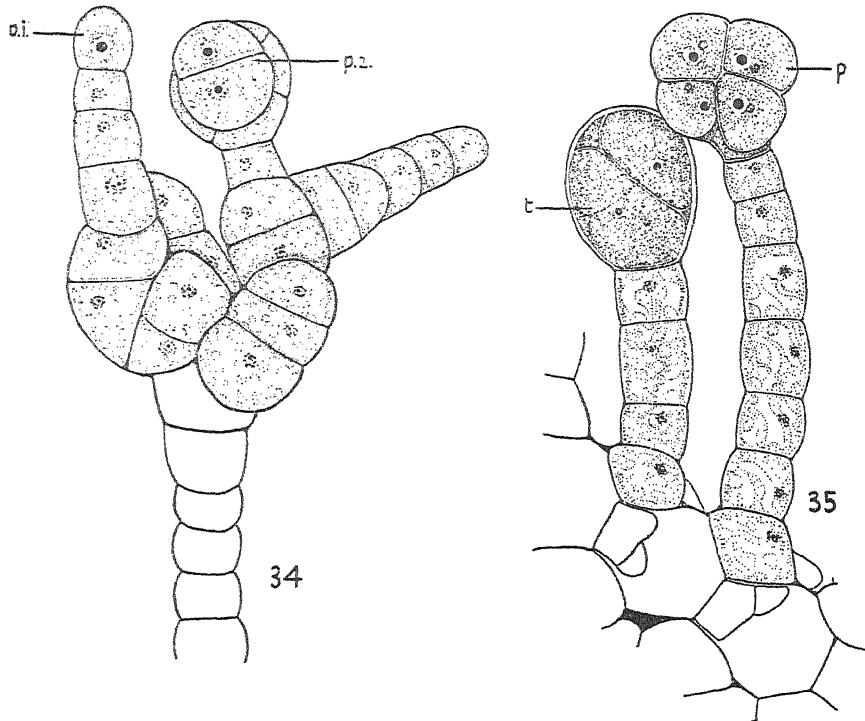
The following table gives a list of collections examined and shows that, unless in a sterile condition, the haploid plants bear sexual organs, the diploid plants tetrasporangia, and the triploid plants parasporangia. Thus the fact is established that the special type of sporangium is formed on the triploid only and the haploid and diploid plants bear only the usual type of reproductive organs.

TABLE

Reproductive organs.	Chromosome number.	Origin of material.
Procarps or cystocarps	Haploid	Dartmouth, Dec. 1936
" " "	"	" Aug. 1937
" " "	"	" Sept. 1937
Spermatangia	"	Ringstead, Dorset, Apr. 1938
Tetrasporangia	Diploid	Dartmouth, Dec. 1936
"	"	Poyllavaish, I.O.M., Apr. 1931
"	"	Dartmouth, Dec. 1936
"	"	" Sept. 1937
"	"	" Jan. 1938
None	"	Scilly Is., Apr. 1936
	"	Chapman's Pool, Dorset, Apr. 1938
Parasporangia	Triploid	Bull Bay, Anglesey, Mar. 1932
"	"	Borth Saint, Anglesey, Mar. 1932
"	"	Lee Bay, Devon, Apr. 1937
"	"	Chapman's Pool, Dorset, Apr. 1938
None	"	Kristineberg, Sweden, July 1937
	"	Chideock Bay, Dorset, Aug. 1937

Having established the fact that haploid, diploid, and triploid plants exist and that the triploid bears a special type of sporangium, it remains to be shown how they are related from the point of view of the life-cycle. The main questions that arise are:

1. Is there cytological evidence for supposing that there is a regular alternation of haploid and diploid individuals as is usual in the diplobiontic Florideae?
2. What is the chromosome complement of the paraspores borne on the triploid plants, i.e. do they give rise to further triploid plants?
3. How does the triploid arise, and what part does it play, if any, in the life-cycle?



TEXT-FIGS. 34-5. Fig. 34. A degenerate parasporangium, cells of which have given rise to three filaments, one bearing a parasporangium initial (*p.* 1) and another a several-celled parasporangium (*p.* 2). $\times 450$. Fig. 35. Portion of an axis bearing a parasporangium (*p.*) and a tetrasporangium (*t.*), on neighbouring branchlets. $\times 450$.

being behind another chromosome and the other behind the nucleolus. Pl. X, Fig. 4, is of a triploid nucleus from the cell of a plant bearing parasporangia. The triploid nuclei are much more difficult to count exactly, for although the nuclear area is usually slightly larger, the chromosomes are more crowded together. Thus in the case of the nucleus figured it is not possible to tell whether three, marked *a*, are single or double, and so the total is ninety or ninety-three. Five of these chromosomes are not figured as they are behind other chromosomes or the nucleolus. The nucleus of an apical cell of a branch of a parasporic plant from Sweden is also figured (Pl. X, Fig. 16). Ninety-two or ninety-three chromosomes are distinguishable in this nucleus, the structure marked *a*, being either one dividing chromosome or, more probably, two very close together. Seven of the total number of chromosomes do not show in the figure as they are under the nucleolus or other chromosomes. It will be noticed that this nucleus is smaller than that of the corresponding English material. This difference is constantly displayed in the material examined, but the reason for it is obscure.

The chromosomes of *P. elegans* differ from those of *Spermothamnion Turneri* and *Spermothamnion Snyderae* in having a greater variety of size and shape. Some are very long, others are two to three times their width and sausage-shaped, but the majority are spherical. Some of the latter are exceedingly small. It has not been possible to determine how many of each type are present, and to check their repetition in the diploid and triploid on account of their very small size and the fact that the appearance of a particular chromosome depends so much on its position. In the haploid set there are probably three very long ones.

The following table gives a list of collections examined and shows that, unless in a sterile condition, the haploid plants bear sexual organs, the diploid plants tetrasporangia, and the triploid plants parasporangia. Thus the fact is established that the special type of sporangium is formed on the triploid only and the haploid and diploid plants bear only the usual type of reproductive organs.

TABLE

Reproductive organs. Procarps or cystocarps	Chromosome number. Haploid	Origin of material.
<i>Spermatangia</i>		Dartmouth, Dec. 1936
" " "	"	" Aug. 1937
" " "	"	" Sept. 1937
Tetrasporangia		Ringstead, Dorset, Apr. 1938
" " "	"	Dartmouth, Dec. 1936
" " "	"	Poylevaish, I.O.M., Apr. 1931
None	Haploid	Dartmouth, Dec. 1936
"	"	" Sept. 1937
"	"	" Jan. 1938
"	"	Scilly Is., Apr. 1936
None	Diploid	Chapman's Pool, Dorset, Apr. 1938
Parasporangia		Bull Bay, Anglesey, Mar. 1932
"	"	Borth Saint, Anglesey, Mar. 1932
"	"	Lee Bay, Devon, Apr. 1937
"	"	Chapman's Pool, Dorset, Apr. 1938
None	Triploid	Kristineberg, Sweden, July 1937
"	"	Chideock Bay, Dorset, Aug. 1937

Having established the fact that haploid, diploid, and triploid plants exist and that the triploid bears a special type of sporangium, it remains to be shown how they are related from the point of view of the life-cycle. The main questions that arise are:

1. Is there cytological evidence for supposing that there is a regular alternation of haploid and diploid individuals as is usual in the diplobiontic Florideae?
2. What is the chromosome complement of the parospores borne on the triploid plants, i.e. do they give rise to further triploid plants?
3. How does the triploid arise, and what part does it play, if any, in the life-cycle?

The major criteria of the normal relationship of the haploid and diploid generations in the diplobiontic Florideae are, firstly, a fusion of a haploid male with a haploid female nucleus to give diploid carpospores, and secondly, a reduction division in the tetrasporangium on diploid plants. As these details have been described so frequently and these processes in the alga of this investigation add nothing essential to the existing knowledge, they will be dealt with as briefly as possible and only in so far as they are relevant to the problems created by the presence of triploid individuals.

In following the nuclear history of the sexual plants, one male and three female plants have been available for study. On the male plant some of the spermatangial branchlets are mature, but in the younger ones dividing nuclei are frequent. In all cases where counts have been made, the haploid number of chromosomes is present. As each spermatangial mother-cell produces two or three spermatangia, the nuclei also divide two or three times, and Figs. 5 and 6 on Pl. X show the prophases of a first and a second division in spermatangial mother-cells. These nuclei, although very small, occupy almost the entire cell. Thirty-one chromosomes are present in both of these nuclei, but two are not visible in Fig. 6.

Only one of the female plants has any wealth of procarps in the early stages of development, and only a few examples of nuclear divisions at the stage when the chromosomes can be counted have been found. Two of these are figured (Pl. X, Figs. 7, 8), and it will be seen that they have the haploid number of chromosomes, but in Fig. 7 one chromosome is hidden behind the nucleolus. Pl. X, Fig. 7, shows the prophase of the nuclear division in the apical cell of a two-celled carpogonial branch, that of Text-fig. 9, and a similar stage in the nuclear division in the apical cell of a three-celled carpogonial branch, that of Text-fig. 11, is shown in Fig. 8. One of the daughter nuclei resulting from this division will become the nucleus of the carpogonium itself.

These facts provide evidence that the sexual plants examined produce haploid spermatia and haploid carpogonia. Pl. X, Fig. 9, shows the nucleus of a carpogonium (that of Text-fig. 14), which appears to have been fertilized as there is a spermatium adhering to the trichogyne, the contents of which have shrivelled and are no longer in connexion with those of the carpogonium. The plant bearing this carpogonium is haploid but the carpogonium nucleus is diploid. The nucleus contains sixty-one chromosomes, six of which are not visible in the figure and one of which, marked *a*, may in reality represent two chromosomes very close together. The long thread-like body, *a*, is probably just outside the nuclear membrane, but it is not possible to be certain.

Investigation of the numbers of chromosomes present in the nuclear divisions between fertilization and the formation of the carpospores is considerably hampered by the great numbers of filaments which grow up around the fertilized procarp and by the thickness of the developing cystocarp itself. The nucleus of a central cell from which one gonimolobe initial has arisen is shown in Fig. 10 on Pl. X. It is in division prior to the cutting off of a

second initial, and sixty-four bodies, five of which are not shown in the drawing, are visible in the nucleus. The nuclei of the cells of the developing gonimolobes are small in volume and surrounded by dense protoplasm. The best example found is shown in Fig. 11 on Pl. X. The diploid number of chromosomes is present, but in the figure seven are not shown as they are immediately beneath others.

With one exception only, all the diploid plants examined bear tetrasporangia, sometimes in great profusion. The tetrasporangium initial is uninucleate and the nucleus undergoes a reduction division and four spores are formed. In late diakinesis thirty-one gemini can be seen. In the example figured (Pl. X, Fig. 12) two of the thirty-one are completely covered by the nucleolus, and it is to be noticed that the individual chromosomes of one pair, *a*, are still separate.

Nothing abnormal, therefore, has appeared in the nuclear history of the haploid and diploid plants examined and the results of the investigation of the parasporangium will now be given.

A glance at Text-figs. 28-33 will show the development of the parasporangium, and for a description the reader is referred to pp. 355-7 in the preceding section. The nucleus of the parasporangium initial is very much larger than that of a vegetative cell, but in the successive divisions some of this increased size is lost again. Several counts of the numbers of chromosomes present in the late prophase of the various nuclear divisions in parasporangia have been made, and in all cases the number is sufficiently near ninety-three to leave no doubt that they are invariably triploid, as are the three examples figured. Fig. 13 on Pl. X is a drawing showing the late prophase of a nuclear division in a one-celled parasporangium, and Figs. 14 and 15 are of similar nuclei in a three-celled and an eleven-celled parasporangium respectively. It is not possible to count the chromosomes present in the last divisions in a maturing parasporangium as the protoplasm is then very dense, but there is no reason to suppose that any alteration in the chromosome number would take place at that stage. From the evidence available it seems justifiable to conclude that the mature spores are triploid like the plants on which they are borne. This being so, they will give rise in their turn to triploid plants, and since the paraspores are produced in large numbers, a reason for the abundance of triploid plants is provided.¹

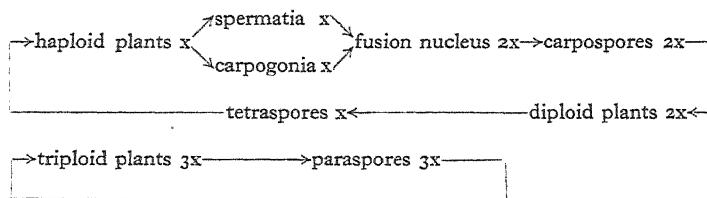
A drawing of one of the nuclei of a five-celled parasporangium on a plant from Sweden is given (Pl. X, Figs. 17 and 17a) to show that they also are triploid. Here the chromosomes number eighty-nine or ninety-one, two structures, marked *a*, representing either one or two chromosomes. As seventeen

¹ As has been stated previously, a few tetrasporangia sometimes develop on parasporic plants. Occasionally the nucleus of the sporangium initial divides and four spores mature, but in many cases the initial degenerates even before the first division of the nucleus. The number of chromosomes present in the case of the nuclei, which do divide, has not been ascertained, and so the chromosome complement of tetraspores formed on triploid plants is unknown.

of the chromosomes are behind the nucleolus or other chromosomes, they are shown separately (Pl. X, Fig. 17a). The Swedish sporangial and vegetative nuclei are much smaller than in the corresponding English material.

With regard to the third question concerning the origin of the triploid and the part, if any, that it plays in the life-cycle, unfortunately no evidence of the method of origin of the triploid plants has accrued during this cytological investigation. The possible ways in which it might originate seem to be, firstly, by a fusion of haploid male and diploid female nuclei or vice versa as a result of the sexual organs developing on diploid individuals as they do frequently in the case of *Spermothamnion Turneri* (Drew, 1934); secondly, the fusion of the diploid fusion nucleus from the carpogonium with the haploid auxiliary cell nucleus; or thirdly, the fusion of two haploid spermatial nuclei with one haploid carpogonial nucleus. During the investigation any evidence of such fusions has been sought, but nothing suggestive of such happenings has been seen. Considering how small a number of sexual plants has been available, this negative evidence cannot be considered final, however. It is hoped that additional sexual plants may be found and examined, though further evidence may well continue to be of a negative nature.

This absence of evidence also means that it is not yet possible to indicate whether the triploid arose once during the history of the species or whether it is still being formed, or define the part played in the life-cycle by the triploid. From the present state of our knowledge it appears that the haploid and diploid generations are related in the usual way and there is no connexion between these and the triploid plants. This can be expressed diagrammatically as follows:



5. DISCUSSION

The cytological part of this investigation has shown that in addition to a normal diplobiontic Floridean life-cycle with an alternation of haploid sexual and diploid asexual generations, *Plumaria elegans* has triploid individuals which reproduce themselves by parasporangia. The presence of these triploid individuals bearing a special type of sporangium is undoubtedly the most interesting fact that has emerged from the investigation. This is the first account, based on nuclear evidence, of triploid plants in the Florideae¹ and as far as the writer knows in the whole of the algae. The possible methods

¹ Triploid carpospores have been reported in *Spermothamnion Turneri* (Drew, 1934) and triploid nuclei in the sporangia of *Spermothamnion Snyderae* (Drew, 1937).

of origin of the triploid have been mentioned already (p. 362), but the fact that it has survived and occurs so commonly now calls for explanation and comment. Its survival is due without any doubt to the fact that it has its own method of reproduction by parasporangia, for without these it would be entirely dependent on being continually formed afresh. The next questions to be raised are how and when the parasporangia arose, but unfortunately nothing can be given in answer to these. However, since the parasporangia never occur on haploid and diploid individuals, it would seem safe to assume that the origin of the parasporangium has been closely connected with that of the triploid itself.

As has been previously stated, occasional tetrasporangia mature on the triploid of *Plumaria elegans*, and this suggests that an attempt at a reduction division is made, but whether it is irregular, as is frequently the case in the triploids of higher plants, is unknown.

The triploid appears to have been able to spread into colder waters than the haploid and diploid and thus has a wider distribution. In this it can be compared with some of the polyploids known in the Angiosperms (Muntzing, 1935-6; Manton, 1934 and 1935). According to Rosenvinge (1923-4) the sexual plants do not occur on the Danish coast, and Kylin (1923) reports the same is true for Sweden, the earlier records of cystocarps having been proved by an examination of the preserved specimens to be parasporangia. Rosenvinge (l.c.) has found only two tetrasporangia in Danish waters and they were on a parasporangia-bearing plant. This writer has found a few tetrasporangia among the parasporangia on triploid plants from Sweden. All plants from Heligoland examined by Pringsheim (1862) bore no other reproductive bodies besides parasporangia. Most of this evidence of the wider distribution of the triploid is based on records of the occurrence of the reproductive organs, but it is hoped that the opportunity will arise for a cytological examination to be made of plants from various localities in the total range of the species. The wider distribution of the triploid suggests that it is not of recent origin.

In any one locality where haploid, diploid, and triploid individuals all occur, the triploids appear to be the most numerous. This may be the result of greater vigour due to the triploid condition or to its very effective method of reproduction by means of paraspores. Evidence regarding these possibilities would be very difficult to obtain, and in any case the number of triploids would have to be compared with the total of haploids and diploids as the triploid is able to reproduce itself directly but the diploid only by the intervention of the haploid and vice versa.

With regard to the morphology of the parasporangia of *P. elegans*, Oltmanns (1922) considered them to be modified tetrasporangia. Rosenvinge (1923-4), on the other hand, did not support this view but did not advance an alternative theory. Kylin (1937), however, states that 'Meiner Meinung nach sind die Parasporen bei *Plumaria* vegetative, diploide Vermehrungskörper, die mit den Tetrasporen nicht homolog sind'. This present investigation has shown that

the parasporangia of *P. elegans* are asexual sporangia of a special type borne on triploid individuals. It would seem very doubtful whether they are homologous with the tetrasporangia, and they certainly have no closer relationship with polysporangia, such as those of *Spermothamnion Snyderae* Farlow.

These facts regarding the parasporangium of *P. elegans* obviously cannot be applied to the other parasporangia of the Ceramiales, which will all have to be investigated separately.

6. SUMMARY

1. *Plumaria elegans* has spermatangial, procarpic, and tetrasporic plants as is usual in the diplobiontic Florideae.

2. In addition a fourth type of plant bearing parasporangia is frequently found. Occasionally a few tetrasporangia develop among the numerous parasporangia.

3. A cytological investigation has shown that plants bearing sexual organs have thirty-one chromosomes and those bearing tetrasporangia sixty-two. The result of sexual fusion is a cystocarp with diploid carpospores and there is a reduction division in the tetrasporangium. The haploid and diploid plants are undoubtedly related in the way usual in the diplobiontic Florideae.

4. The plants which bear parasporangia (with rare tetrasporangia) have ninety-three chromosomes and are therefore triploid. No change in the chromosome number accompanies the formation of parospores, which like the parent plant have ninety-three chromosomes and are triploid. No evidence has yet accrued to show whether there is any relationship between the haploid and diploid plants on the one hand and the triploid plants on the other. This is apparently the first cytological evidence of triploid plants in the algae.

5. There is no evidence yet available to show how the triploid originated. The fact that the formation of the triploid has been accompanied by the development of a new type of reproductive body, the parasporangium, is without any doubt the reason for its survival. There is a certain amount of evidence which indicates that the triploid has a wider geographical distribution than either the haploid or the diploid.

6. The fate of the occasional tetraspores borne on the triploid and their chromosome complement are facts still to be ascertained.

7. While both parasporangia and tetrasporangia arise from uninucleate initials, the similarity between the two sporangia ends there. In contrast to the tetrasporangium where there is a very orderly division of the protoplast after the two nuclear divisions are complete, the divisions in the parasporangium are entirely fortuitous and a cell division follows every nuclear division, of which there are several.

The writer wishes to express her indebtedness to those who have assisted by the collection of material, Professor H. Kylin, Dr. B. Colson, Miss E. Jackson, and especially Dr. I. Manton. She also wishes to record her thanks

to Professor W. H. Lang for his interest and help and to the Council of the Victoria University of Manchester for laboratory facilities. To Miss C. I. Dickenson she offers her thanks for investigating questions of nomenclature.

POSTSCRIPT

Mention should be made of a paper 'Über die Entwicklungsgeschichte von *Plumaria elegans*' by Suneson (1938), which has been received since most of the preceding account was written. As no investigation of the nuclear condition was made by Suneson, the parasporangia are still regarded by him as reproductive bodies regenerating the diploid generation. The difference of the reproductive cycle on the Swedish coasts and the coasts of Brittany is remarked on, and a short account is given of the development of the parasporangia, tetrasporangia, and procarps. Spermatangia and developing cystocarps were not seen.

With regard to the morphological questions, a very early stage in the development of the procarp is the only one where there is not complete agreement between the two accounts. While Suneson asserts that the carpogonial branch initial is always cut off before the sterile cell, the present writer has seen procarps in some of which the sterile cell is formed first, and in others the carpogonial branch initial (see p. 350), the two conditions occurring on the same axis.

The present paper shows how essential is the cytological information for a correct understanding of the nature of the parasporangium, of the reproductive cycle as a whole and also of the ecological differences.

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EXPLANATION OF PLATE X

Illustrating Kathleen M. Drew's paper on 'An Investigation of *Plumaria elegans* (Bonnem.) Schmitz with Special Reference to Triploid Plants bearing Parasporangia'.

The nuclei figured on this plate are all in late prophase. $\times 4,200$. These drawings are made by superimposing camera lucida tracings of the chromosomes of successive focal planes. In order to suggest their position in a sphere, the chromosomes are variously shaded, those nearest the observer being black and those farthest away being white. Where chromosomes are completely behind the nucleolus or other chromosomes no attempt is made to show them except in the special case of the nucleus of Fig. 17, where they are so numerous that they are shown separately in Fig. 17a.

Fig. 1. Nucleus of apical cell of male plant. Thirty-one chromosomes present, but one not shown as behind nucleolus.

Fig. 2. Nucleus of apical cell of female plant. Thirty-one chromosomes present, two of which are not shown, being behind the nucleolus.

Fig. 3. Nucleus of apical cell of tetrasporic plant. Sixty-two chromosomes present, one of which is behind the nucleolus and a second is behind another chromosome.

Fig. 4. Nucleus of apical cell of plant bearing parasporangia. Each of three structures marked *a* may represent one dividing chromosome or two very near together. Total number of chromosomes ninety or ninety-three. Four of these chromosomes are behind the nucleolus and a fifth is behind another chromosome.

Fig. 5. Nucleus of spermatangial mother-cell, prior to first division. Thirty-one chromosomes distinguishable.

Fig. 6. Nucleus of spermatangial mother-cell which has already cut off one spermatangium. Thirty-one chromosomes present, two of which are behind the nucleolus.

Fig. 7. Nucleus of apical cell of carpogonial branch of Text-fig. 9. Thirty-one chromosomes present, but one hidden behind nucleolus.

Fig. 8. Nucleus of apical cell of carpogonial branch of Text-fig. 11. Thirty-one chromosomes present.

Fig. 9. Fusion nucleus in carpogonium of Text-fig. 14. Long thread-like body, *a*, is probably extra-nuclear. Other structure marked *a* may represent one or two chromosomes, bringing the total to sixty-one or sixty-two. Six of these, however, are not shown in the figure as they are behind others.

Fig. 10. Nucleus in central cell after one gonimobote initial has been formed. Sixty-four bodies distinguished in nucleus, five of which are not shown in figure.

Fig. 11. Nucleus of cell of gonimobote. Sixty-two chromosomes present, seven of which are behind others and so are not shown in the figure.

Fig. 12. Nucleus of a tetrasporangium initial, in late diakinesis. Thirty-one gemini present, two of which are behind the nucleolus. In the case of one, *a*, the individual chromosomes are still visible.

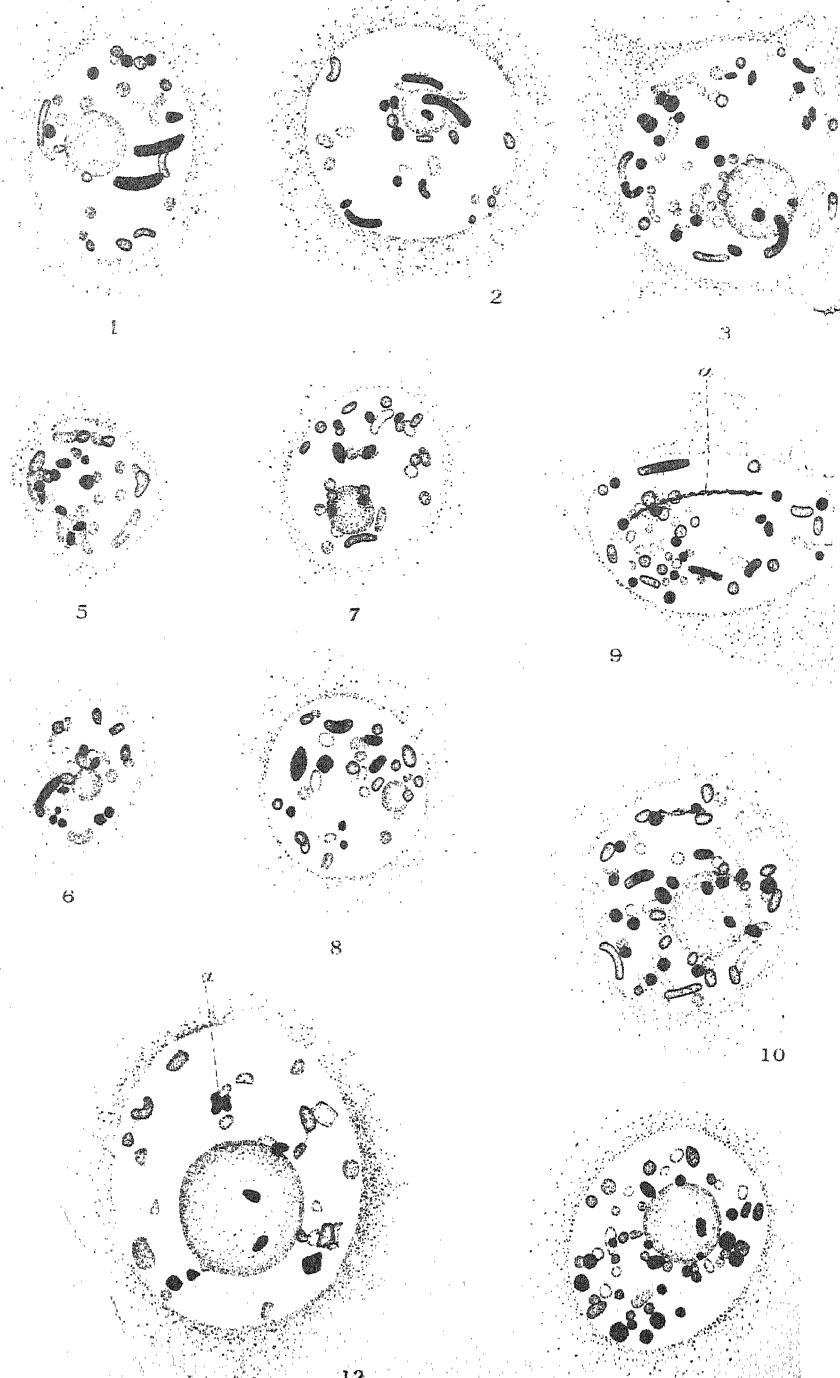
Fig. 13. Nucleus of an undivided parasporangium. Ninety-two chromosomes distinguishable, but two (one of which is marked *a*, and the other is behind the nucleolus) may be double. Eighty-nine total figured.

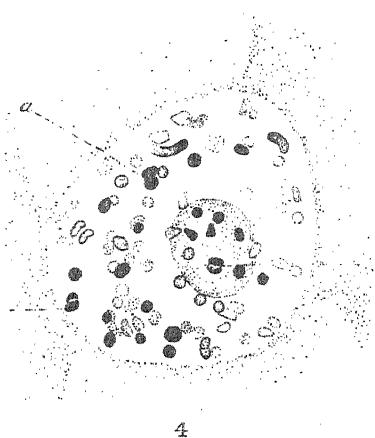
Fig. 14. One of nuclei of a three-celled parasporangium. Ninety-one chromosomes present, two of which marked *a*, may in reality be two chromosomes near together. Nine are not figured as behind nucleolus or other chromosomes.

Fig. 15. Nucleus of an eleven-celled parasporangium. Ninety-one or possible ninety-three chromosomes present. Each of structures marked *a* may represent one dividing chromosome or two close together. As seven are behind the nucleolus or other chromosomes, they do not appear in the figure.

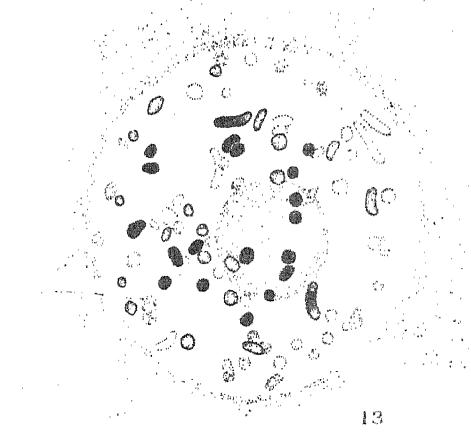
Fig. 16. Nucleus of apical cell of plant from Sweden, bearing parasporangia. The chromosomes total ninety-two or ninety-three, one marked *a*, being in all probability two very close together. Seven chromosomes are completely behind others.

Figs. 17 and 17a. Nucleus of a five-celled parasporangium on plant from Sweden. Each of structures marked *a* may be one dividing chromosome or two close together, bringing total to eighty-nine or ninety-one. Seventeen, which are behind either the nucleolus or other chromosomes, are shown separately in Fig. 17a.

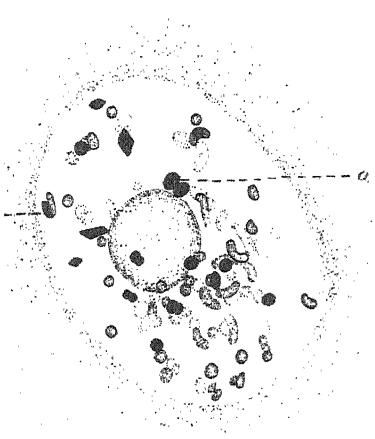




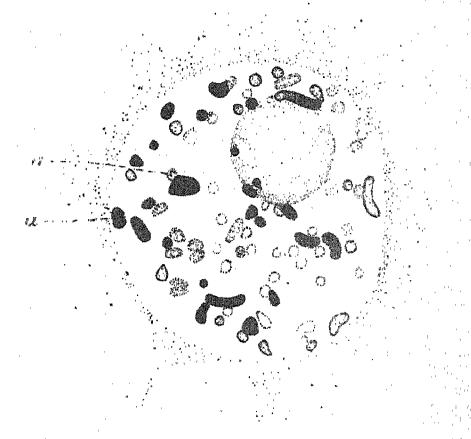
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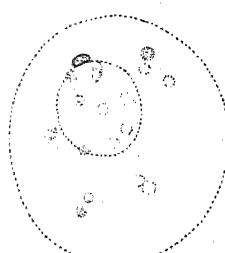
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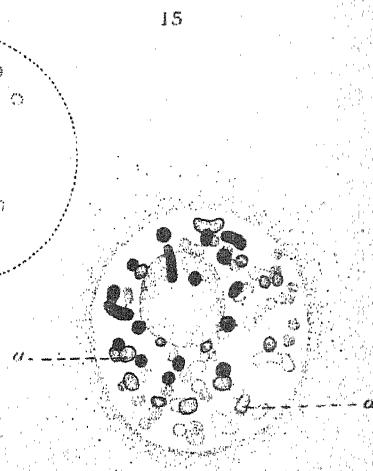
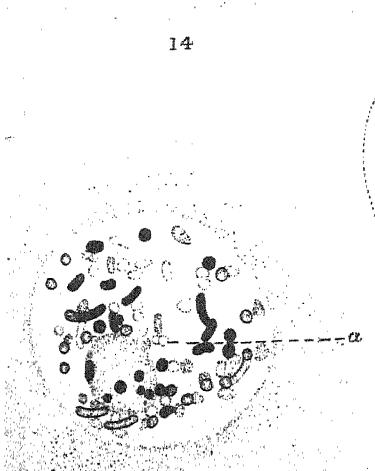
14



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17a



A Modified Technique for the Microscopic Examination of the Xylem of Whole Plants or Plant Organs

BY

E. M. DEBENHAM

With Plate XI

INTRODUCTION

As an aid to anatomical investigation an attempt was made to clear whole plants with a view to elucidating the course and structure of the xylem. Permanent preparations were desirable and the technique employed was that described by K. Barratt (1920), Eau de Javelle being the clearing medium and ammoniacal fuchsin the xylem stain. Good results were obtained with whole plants of *Phylloglossum Drummondii*. Subsequently, the technique was repeated with a large variety of material including flowers, seedlings, and fern fronds. The results obtained showed that material which could be cleared easily would stain satisfactorily. Material with abundant food storage or a deep pigmentation such as large cotyledons and hypocotyls of seedlings and the older fern petioles required a period of clearing exceeding one month, and frequently the constitution of the lignin was so affected as to cause it to take the ammoniacal fuchsin very feebly or not at all. In addition such material was extremely fragile. However, the successful results obtained showed that good preparations would be of value not only in anatomical research, but also for teaching purposes and an attempt was made to find a more rapid clearing fluid.

J. L. S. Simpson (1929) employed 75 per cent. aqueous lactic acid as the clearing fluid and obtained permanent but unstained preparations by killing clearing and mounting in the medium, a clearing period of 2-12 hours in an oven at 54° C. being sufficient for the material under consideration.

This method was repeated and stained permanent preparations were obtained with ammoniacal fuchsin and were mounted in Canada balsam. The sole disadvantage of this method appeared to be the inability of the lactic acid to bleach the material, but this was subsequently remedied by the use of Eau de Javelle for a short period after clearing. The advantage lay in the comparatively rapid, but thorough clearing while the material did not become so fragile. Only the un lignified or partially lignified elements in a number of different plants failed to take the brilliant red colouration.

THE CLEARING AND STAINING PROCESS

i. *Preparation for clearing.*

Fresh or dried herbarium material or material fixed in 70 per cent. alcohol may be used. The fresh and alcohol-preserved material may be transferred to the clearing fluid. Dried material should first be soaked in water at a temperature of 60° C. for not less than 3 weeks, the period depending on the thickness of the material.

ii. *Clearing and washing.*

The material is placed in 70–75 per cent. lactic acid in corked tubes or in covered Petri dishes and kept in an oven at a temperature of 58–60° C. until clear. A period of 1 week is often sufficient, but as long a time as 3 weeks may be employed without detriment to thicker material. In the course of a long period the lactic acid should be replaced with fresh.

When cleared the material is removed from the oven, the fluid poured or pipetted off and replaced by 70 per cent. glycerine in the cold. The glycerine should be changed at least once in a period of 2–3 days. The cleared objects in tubes are now transferred to covered Petri dishes and all further operations are carried out in those dishes so that handling of the material is reduced to a minimum. Waste fluids are pipetted from the dishes and the replacing liquids poured or pipetted in with care. Not more than two large or five small objects are placed in each dish, otherwise damage may be caused during the final mounting.

A further washing in 50 per cent. glycerine for 24 hours followed by changes of 50 per cent. alcohol for 2–3 days should remove all traces of lactic acid. If not required immediately the material is stored in 70 per cent. alcohol or in Calberla's Fluid after treatment with 70 per cent. alcohol.

iii. *Preparation for bleaching.*

The 50 per cent. alcohol is pipetted off and replaced by descending grades of alcohol and finally water. Material which has been stored in Calberla's fluid is first given a thorough washing in changes of 70 per cent. alcohol for 6–24 hours.

iv. *Bleaching and washing.*

Examination of apparently colourless material with a microscope shows that lactic acid fails to remove all pigmentation and that a separate bleaching is necessary.

For this purpose a strong solution of Eau de Javelle (Zimmermann, 1893) is obtained by allowing bleaching powder to dissolve in water for at least 24 hours, filtering off the undissolved solid, and adding a fresh concentrated solution of potassium oxalate in distilled water until no further precipitate is formed. The Eau de Javelle may be kept in a fresh condition over this precipitate and filtered off as required.

The water is pipetted off from the dishes and replaced by Eau de Javelle. The dishes are then placed on a hot plate or oven at a temperature of about 40° C. and kept under observation until the material is decolourized. The process may be complete in 20 minutes or may take several hours.

Thorough washing in several changes of cold distilled water for not less than 12 hours is necessary and a final treatment with 10 per cent. alcohol is given before staining. Material may be stored at this stage by passing up the graded alcohols to 60 per cent. alcohol and thence to Calberla's fluid. Even a storage in this liquid for 48 hours may be of advantage in tending to remedy any shrinkage caused by the bleaching process. A thorough washing in several changes of 70 per cent. alcohol for 6-12 hours then follows and a descent of the graded alcohols to 10 per cent. alcohol before staining.

Should very light material float after the bleaching process under no circumstances should it be forced beneath the surface of a liquid, but should be kept well covered and free from dust.

v. *Staining.*

The 10 per cent. alcohol is pipetted off and replaced by freshly made ammoniacal fuchsin. The solution used is a modified form of that described by Zimmermann (1893) and is prepared by the addition of 0.880 ammonia solution from a dropping funnel to a flask containing a small quantity of a filtered saturated solution of Gurr's 'Special' basic fuchsin in absolute alcohol. The ammonia is allowed to flow in a slow stream, the flask being shaken all the time until the liquid shows a pale yellow colour. In this way, precipitation is avoided and filtration is unnecessary. The stain should not be allowed to stand longer than 24 hours before using.

The material should lie well covered in the stain for 1-3 days, the time depending on the thickness of the material. The dishes are kept in a cool place so that loss of ammonia is not so rapid as to cause the reappearance of the red colour in the liquid.

vi. *Washing and dehydration.*

On the completion of the staining period the ammoniacal fuchsin is pipetted off and the material treated with absolute alcohol. Two or three changes may be necessary before the red stain restored by the alcohol treatment is washed out from the thin-walled tissue and sclerenchyma. The xylem is stained a brilliant red where lignification is sufficiently advanced. A final treatment with absolute alcohol is given for dehydration.

vii. *Mounting.*

(a) *To mount thin materials which do not require a cell.* Thin objects are floated on to a clean slide placed in the Petri dish of absolute alcohol and the slide with the material is lifted out and washed over with absolute alcohol. The liquid is then quickly drained off and the object is mounted in euparal,

a mountant which gives great transparency where comparatively few layers of thin-walled tissue surround the wood. Small bubbles may be removed if the slide is supported horizontally over a hot plate.

(b) *To mount thicker objects.* Such material requires to lie in a rectangular cell. This can be made by cutting thin strips of glass from a slide or photographic plate to the required lengths and fixing them to a clean slide with a film of thin Canada balsam. The balsam is allowed to dry by keeping the slide on an oven or hot plate for at least 12 hours before using. For the thickest objects two or even three layers of glass strips are necessary to give a sufficiently deep cell.

Canada balsam is found to be the more suitable mounting medium for objects requiring any but the shallowest of cells. Euparal has the advantage of producing great transparency while showing up cellulose walls owing to the low refractive index as compared with Canada balsam. In a relatively thick mount such as the seedling of *Cucurbita Pepo* the intervening walls of the cortical and pith cells are very numerous and the transparency as regards examination of the xylem is much reduced when Euparal is employed. For such material Canada balsam may be used after treatment with cedarwood oil. The absolute alcohol is pipetted from the dish and the material is allowed to lie in the oil until it is thoroughly impregnated as seen by the complete transparency. The slide is then placed in the dish and the material floated into the cell. On removal from the dish the oil is drained away until the surface of the specimen is almost dry. The cell is then completely filled with Canada balsam to allow of a film spreading over the edges of the cell and a coverslip is added. The Canada balsam should be just liquid enough to pour so that it will dry more quickly and not tend to loosen the sides of the cell. Any small bubbles can be removed by supporting the slide horizontally over a hot plate.

The accompanying photomicrographs (Pl. XI) of cleared preparations were selected to show something of the range of application of the above technique.

SUMMARY

A modification of a clearing technique for uncut material is described together with the subsequent staining and permanent mounting. The object of such treatment is the elucidation of the course and structure of the xylem in whole plants or plant organs.

The method has been successfully employed with a variety of vascular plants. It involves (1) clearing in 70–75 per cent. lactic acid at 58–60° C.; (2) bleaching in Eau de Javelle at 40° C.; (3) staining the xylem with ammoniacal fuchsin; (4) mounting in euparal or Canada balsam.

In conclusion I desire to express my thanks to Dr. H. Duerden for his kind supervision of this work and to Professor Dame Helen Gwynne-Vaughan for much valued criticism.

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- SIMPSON, J. L. S., 1929: A Short Method of Clearing Plant Tissues for Anatomical Studies. Stain Tech., iv. 131.
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EXPLANATION OF PLATE XI

Illustrating Miss E. M. Debenham's paper 'A Modified Technique for the Microscopic Examination of the Xylem of Whole Plants or Plant Organs'.

Fig. 1. *Phylloglossum Drummondii*. Leaf tip showing spirally thickened xylem elements. From a mount of whole plant. $\times 37$.

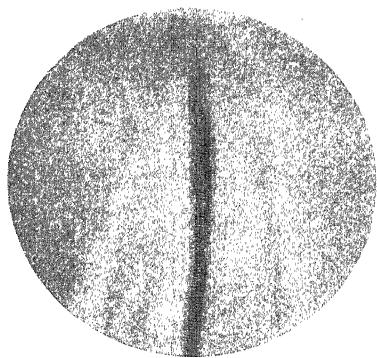
Fig. 2. *Aquilegia sp.* Node of seedling from mount of whole plant. A large lateral xylem strand supplies each cotyledon. The paired intermediate strands with a median trace supply the first foliage leaf. $\times 37$.

Fig. 3. *Aquilegia sp.* One root of seedling from mount of whole plant showing the origin of a young branch root. The diarch condition of each is visible. $\times 150$.

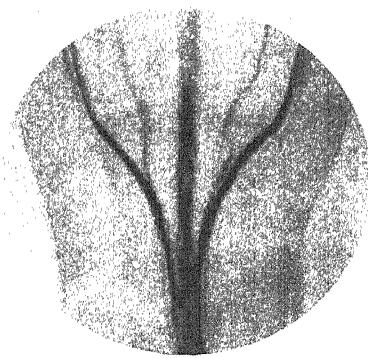
Fig. 4. *Lathyrus odorata*. Base of style from a mount of the flower with corolla removed. Anastomosing xylem traces connect one of the marginal strands on the left with that of the mid-rib. $\times 28$.

Fig. 5. *Osmunda regalis*. Portion of fertile spike. Long thin xylem elements characterize the main traces while those of the branches supplying the sporangia are generally shorter and wider. $\times 37$.

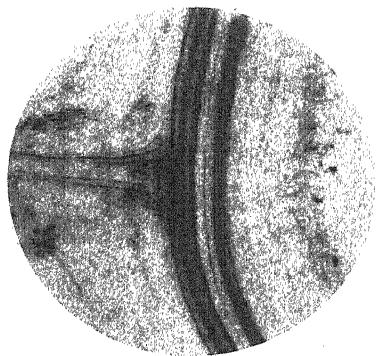
Fig. 6. *Selaginella Wildenovii*. Portion of the stem with bases of ventral leaves. The leaf traces within the stem are very slender, expanding below the base of the ligule. $\times 37$.



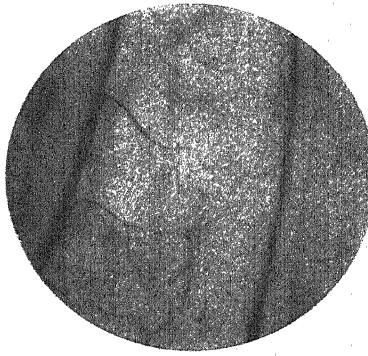
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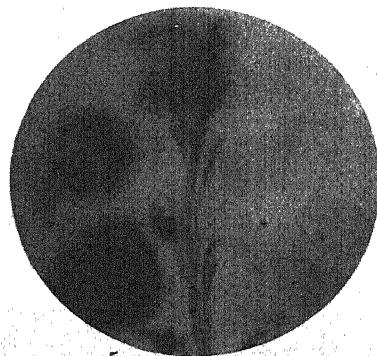
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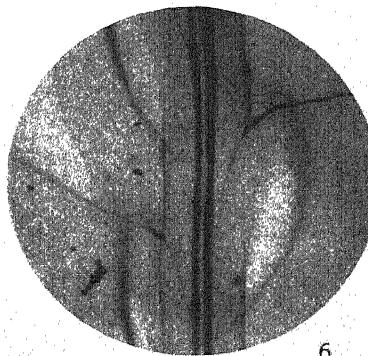
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The Morphology, Cytology, and Alternation of Generations
in *Enteromorpha compressa* (L.) Grev. var. *lingulata*
(J. AG.) Hauck.

BY

K. R. RAMANATHAN

With seventy-four Figures in the Text

THE first cytological study of Ulvaceae was that of Carter (1926) on *Ulva lactuca* L., *Monostroma latissimum* (Kütz.) Wittr., and *Monostroma Grevillei* (Thur.) Wittr. var. *Vahlii* (J. Ag.) Rosenv. She described the details of nuclear division in the vegetative and reproductive cells and showed that *Monostroma latissimum* (Kütz.) Wittr. is dioecious. The gametes from any one plant fail to conjugate among themselves, producing zygotes only when brought into contact with gametes of a plant of the opposite sex. They exhibit slight anisogamy.

Three years later Föyn (1929, 1934b) investigated the life-history of *Ulva lactuca* at Naples and found that the plants produced either zoospores or gametes; the sexual plants were again dioecious. The germlings grown from the zoospores were haploid and produced only gametes, while those grown from the zygotes were diploid and produced only zoospores. Reduction took place during zoospore-formation. In the life-history of *Ulva lactuca* there exists therefore a regular alternation between a haploid sexual and a diploid asexual generation.

Hartmann (1929) simultaneously showed that in *Enteromorpha compressa* (L.) Grev. and in *E. ramulosa* (Eng. Bot.) Hook. there are three different kinds of plants, viz. male, female, and asexual, all externally alike. He suggested, therefore, that *Enteromorpha* had an alternation similar to that established for *Ulva lactuca*. In the following year Kylin (1930) demonstrated the same differentiation in *E. intestinalis* (L.) Link and showed that the conjugating gametes were distinctly anisogamous. Miyake and Kuneida (1931), studying forms of *Monostroma*, *Enteromorpha*, and *Ulva* from the coasts of Japan, observed conjugation of gametes in all instances and confirmed the observations of previous workers, especially as regards sexual differentiation.

Bliding (1933), by means of extensive laboratory cultures, investigated the life-history of various Swedish species of *Enteromorpha* and demonstrated in this genus also a regular alternation of a sexual with an asexual generation.

Although our present knowledge regarding the life-history of *Enteromorpha* is thus fairly complete, the cytological details have so far not been elucidated.

In view of the common occurrence of Enteromorpha in Madras it was decided to investigate the nuclear features of various phases of the life-history, with special reference to the alternation of generations.¹

MATERIAL AND METHODS

The material used in the present investigation was collected in different seasons over a two-year period from the estuarine region of the River Cooum at Madras. The alga occurs here fairly commonly throughout the year and in certain seasons it is found abundantly attached to stones. Collections were usually made late in the afternoon, but occasionally also in the morning. Afternoon collections proved more suitable, since such material not only showed abundant nuclear division the following night, but also produced plentiful swarmers the next morning. The material was transferred to the laboratory in large glass troughs filled with estuarine water.

Material collected in the evening was fixed at hourly intervals during the next twenty-four hours. Some was also fixed at half-hourly intervals during the night, since certain division stages followed one another very rapidly. The most abundant nuclear figures were found in material fixed between 8.30 p.m. and 1 a.m.

The fixatives employed included Flemming's weak and strong solutions, Flemming's strong diluted with an equal amount of estuarine water, Chamberlain's Chicago chrom-osmo-acetic mixture, Navaschin's fluid, Schaudinn's sublimate-acetic-alcohol, and all the various modifications of Bouin's picro-formol mixtures. The best results were obtained with the original Bouin's fluid, as well as with Allen's modification of the same, having the composition picric acid (saturated aqueous solution), 75 c.c.; commercial formalin, 15 c.c.; glacial acetic acid, 10 c.c.; urea (crystals), 1 gm. The material fixed in picro-formol mixtures was usually left in the fluid from a few minutes to twenty-four hours, but even a longer treatment did not show any bad effects. It was subsequently washed in several changes of 70 per cent. alcohol until all traces of picric acid were removed. In the case of mixtures containing chromic acid, the material was left in the fluid from three to nearly twenty-four hours. It was then thoroughly washed, first in a few changes of estuarine water, then in equal amounts of estuarine and tap-water, and finally in running tap-water.

After washing, the material was either directly stained and mounted or it was taken up through a series of alcohols and xylol, embedded in paraffin (m.p. 56–58° C.), and cut into sections 3–10 μ thick. In the former case, small portions of the tubular thallus, opened out with a pair of needles, were stained, dehydrated with glycerine and alcohol, and finally mounted in Venetian turpentine or Canada balsam. Another method employed in making preparations *in toto* was that of Carter (1926) using a slide smeared with a thin layer of Mayer's albumen fixative flooded with water, on which a small portion of the frond, spread out as before, was floated. The slides were then allowed to

¹ A preliminary account was published in 1936 (Ramanathan, 1936).

dry and the material stained direct on the slide. Such preparations showed no shrinkage of the cells and were in all respects far superior to those made by the other method.

Among the various stains used, Heidenhain's iron-alum haematoxylin proved to be the best, though some very good preparations were obtained with Mayer's haemalum. For staining with iron-alum haematoxylin, the following schedule proved the most satisfactory: (1) Bring down the material to water and wash in running water for 10 minutes; (2) mordant in 4 per cent. iron-alum (aqueous solution) 1 hour; (3) wash in running water, 10 minutes; (4) rinse in distilled water; (5) transfer to $\frac{1}{4}$ per cent. aqueous solution of Heidenhain's haematoxylin and stain for 12 hours overnight; (6) wash in running water, 10 minutes; (7) destain in a saturated aqueous solution of picric acid, until proper differentiation is secured; (8) wash in running water, 1 hour; (9) dehydrate through a series of alcohol grades consisting of 10, 20, 30, 40, 50, 60, 70, 85, 95, and 100 per cent. alcohols, about 10-15 minutes in each; (10) clear in clove oil and xylol, and (11) mount in neutral balsam.

In the case of Mayer's haemalum, the formula and method suggested by Chamberlain (1933) was used. This was very useful in studying nuclear details, since it leaves the other cell contents unstained or only feebly stained.

For cultures I followed the methods usually employed for marine algae (Pringsheim, 1921; Kufferath, 1929). The thalli were washed in several changes of filtered estuarine water, using a fine camel-hair brush to remove as far as possible the adhering foreign matter, and were then thoroughly rinsed in several changes of sterilized estuarine water. The plants were then placed singly in dishes. Within a few minutes a cloud of swarmers escaped, which were drawn off with a sterilized capillary pipette and freed from all foreign organisms by allowing them to accumulate on the illuminated side of dishes containing sterilized estuarine water. A single drop containing a few swarmers was placed on a clean slide, and after the swarmers had come to rest, the slides were finally rinsed in a few changes of sterilized estuarine water and placed vertically in small jars containing the culture solution.

The solutions first used were those of Schreiber (1928) and Kylin (1933, p. 3), but the growth of the alga was not satisfactory. The 'Erdschreiber' solution used by Föyn (1934a) proved far more satisfactory, and during the later portion of this investigation was exclusively employed. For preparing this solution, the filtered estuarine water was sterilized in an autoclave at 15 lb. pressure for half an hour. To every 1,000 c.c. of this there were added 50 c.c. of sterilized soil decoction in which 0.1 gm. of sodium nitrate and 0.2 gm. of sodium hydrogen phosphate were dissolved. The solution was well aerated by shaking. The solutions were renewed once a week or once a fortnight.

The cultures were set up in sterilized cylindrical glass jars, 10 cm. high and 7 cm. in diameter, with circular glass covers. Each jar contained about 200 c.c. of the culture solution and the jars were enclosed in a glass chamber in a well-

illuminated veranda, not exposed to direct sunlight. The slides bearing the germlings were removed for examination from time to time, or were fixed by dipping into a jar of Allen's modification of Bouin's fluid. After washing in 70 per cent. alcohol they were taken down to water and stained in iron-alum haematoxylin and after dehydrating and clearing, mounted in Canada balsam. Larger plants were detached from the slides and treated like those collected in nature. The material cultivated in the laboratory showed stages of nuclear division at all times of the day, although these were usually more abundant during the night.

GENERAL MORPHOLOGY AND LIFE-CYCLE

The Enteromorpha studied possesses more or less compressed principal fronds, 5–30 cm. in length and 3–6 mm. in breadth, which are generally branched. The branches are mostly simple and elongate, being generally broader towards the top and gradually attenuated towards the base; they are usually confined to the basal region. The degree of branching and the general aspect of the alga varies very much from season to season, but in view of its general characteristics it has been provisionally determined as *E. compressa* (L.) Grev. var. *lingulata* Hauck.

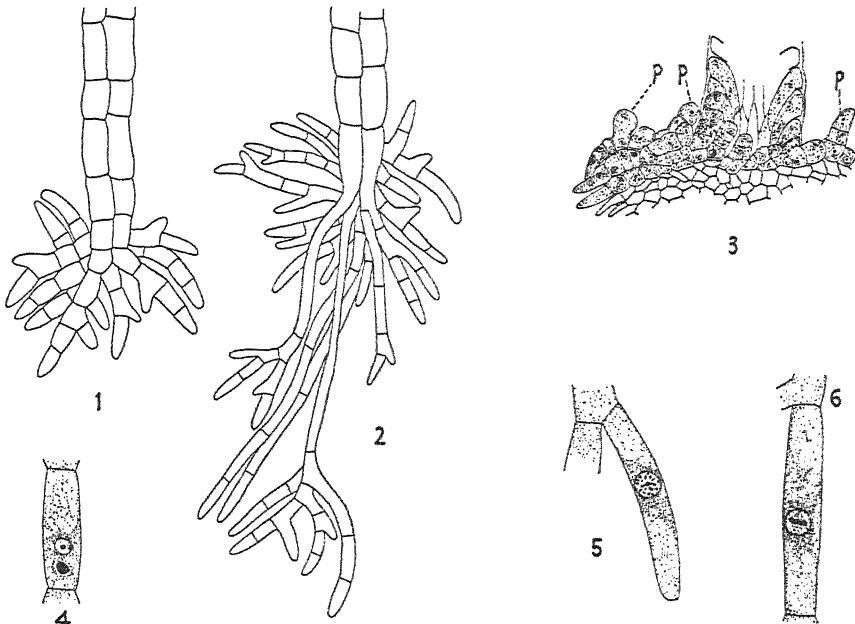
The cells are mostly rectangular to polygonal in surface-view and are usually arranged without definite order, although in certain portions of the frond, especially in young germlings, they show a tendency to be in rows. They measure 10–15 μ by 8–12 μ in surface-view and are 15–25 μ deep in a cross-section of the thallus. The single plate-like chloroplast is usually affixed to the outer wall; there are two to three pyrenoids in each chloroplast in some plants and four to six in others (Figs. 7, 9, 23). The nucleus is located close to the inner wall of the cell. In a piece of thallus, slit open and spread out flat on a slide, all the chloroplasts often come into view at one focus, while all the nuclei are seen at another so that when the thallus is mounted with its inner side upwards, the details of nuclear division are easily observed.

In a mature plant four regions are usually distinguishable: (1) an attaching holdfast, (2) a short narrow stalk, (3) a shorter or longer terminal swarmer-producing region, and (4) a rather long growing region, between (2) and (3). These regions are not sharply demarcated but grade gradually into one another.

The holdfast is composed of a number of living, uninucleate, closely packed cells forming a parenchymatous disc-like expansion (Figs. 1–3), and arising generally by a series of divisions from the primary rhizoidal cell of the germling. Delf (1912) in *Ulva latissima* found the cells of the holdfast to be multinucleate, although Carter (1926) in *Monostroma Grevillei* var. *Vahlii* describes them as uninucleate. The cells of the holdfast frequently proliferate and produce upright filaments, which finally develop into mature fronds. The mode of proliferation was easily followed in plants grown on glass slides in the laboratory. Some of the cells enlarge upwards, the projection is cut off by a cross wall (Fig. 3), and the upper cell divides to form a short filament,

whose further development to form an erect thallus is similar to that of the germling. Proliferation from the holdfast leads to the tufted growth of the plants.

Growth in *Enteromorpha* is intercalary, the growing region where the cells are actively dividing being situated immediately above the stalk. In the



FIGS. 1-6. Structure of the holdfast in *Enteromorpha compressa* var. *lingulata*. Fig. 1. Holdfast of a germling 10 days old ($\times 250$). Fig. 2. Holdfast of a germling 15 days old showing septate and branched prolongations ($\times 300$). Fig. 3. Small portion of holdfast of mature zygotic plant showing origin and development of the proliferations ($\times 245$). Fig. 4. Cell of holdfast with resting nucleus. Fig. 5. Late prophase with diploid number of chromosomes ($2n = 20$). Fig. 6. Metaphase with centrosome-like body at each pole and intranuclear spindle. (Figs. 4-6 $\times 1,200$.)

terminal fertile region every cell forms swarmers which escape through a small round opening in the outer wall. After the escape of the swarmers, the empty part of the thallus persists for a time as a collapsed transparent ribbon, but eventually breaks off. The activity of the cells of the growing region not only makes up for this, but also actually adds to the length of the frond.

In germlings and young plants there is a conical apical portion consisting of one or more cells which do not produce swarmers. After the escape of the first set of swarmers, this apical part is shed with the emptied part below. The swarmer-producing region, which appears terminal in the mature plants, is thus really sub-terminal in origin. The same sterile apical region is found at the ends of the young branches although lost soon after swarmer-formation. After the first crop of swarmers has been liberated, the thallus of course

becomes an open tube, with the characteristic truncated appearance of Enteromorpha.

Two kinds of swarmers are produced by the plants collected in nature, quadriflagellate zoospores and biflagellate gametes, which are, however, always formed on different plants. The two kinds of plants grow together and are indistinguishable from one another. Specimens collected in the evening and kept isolated in the laboratory in separate culture dishes form plenty of swarmers the next morning, when asexual and sexual plants can be separated. They continue to form swarmers of the same kind at intervals of two or three days.

Fusion only takes place when gametes from different plants are brought together. Moreover, fusion only occurs when gametes from certain pairs of plants are mixed, while those from other pairs do not conjugate. In the latter case the plants are evidently of the same sex. *E. compressa* var. *lingulata* is distinctly dioecious. The conjugating gametes are usually slightly unequal in size; the larger ($5\cdot9$ – $6\cdot8$)– $7\cdot8\mu$ long and $2\cdot8$ – $3\cdot9\mu$ broad) are regarded as female and the smaller ($4\cdot9$ – $5\cdot9\mu$ long and $1\cdot8$ – $2\cdot8\mu$ broad) as male. The two types of sexual plants thus distinguished, always produced the same type of gamete on every occasion. Male, female, and asexual plants were investigated cytologically; the male and female plants were found to be haploid, the asexual plants diploid.

Zoospores and zygotes both developed with equal rapidity in the 'Erd-schreiber' solution and the resulting germlings grew into mature plants, which within a month of starting the cultures became fertile and produced swarmers. The germlings developed from zygotes always gave rise to quadriflagellate asexual swarmers, while the germlings from zoospores always gave rise to biflagellate gametes. The plants grown from zoospores included both male and female plants. The life-cycle of the alga was followed through three generations with similar results. The cultural study thus shows that this Enteromorpha possesses a regular alternation of a sexual with an asexual generation, similar in all respects to that described by Hartmann (1929) and Bliding (1933) in other forms of Enteromorpha, and by Föyn (1929, 1934b) in *Ulva lactuca*.

CYTOTOLOGY AND DEVELOPMENT

(a) Somatic mitosis in the gametophyte.

Both the gametophytic plants isolated by the method already described (p. 377) and the plants collected direct from nature showed abundant nuclear division, especially in the growing region of the thallus. In fixed and stained preparations the swarmer-producing region appears much more deeply stained than the growing region, which is evidently due to the denser protoplasmic content of the former, obvious even in unstained specimens. In the growing region nuclear division takes place at the same time in all the cells, so

that all stages of nuclear division are easily obtained. Vegetative mitosis in general occurs between 8 and 11 p.m., the most abundant nuclear figures being found in material fixed at about 9.30 p.m., but occasional division-figures can still be met with as late as 3 or 4 a.m.

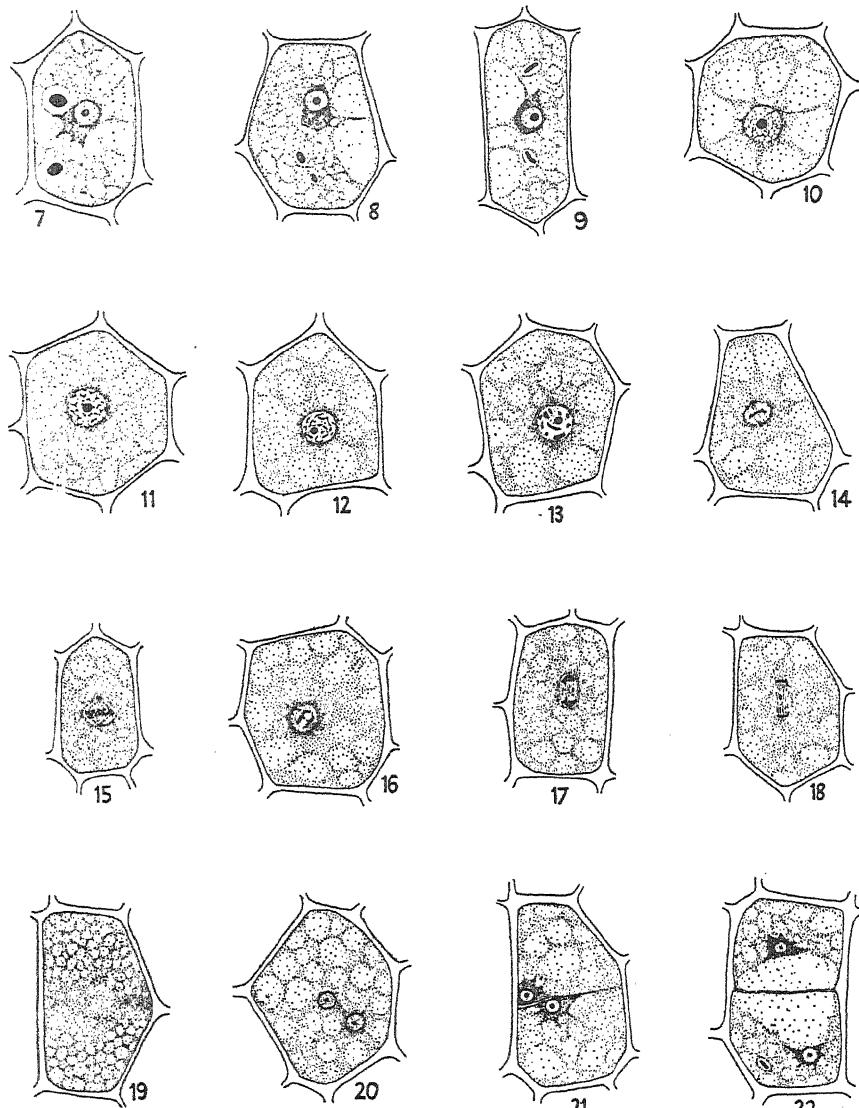
Each resting cell of the gametophyte contains a single nucleus, approximately 2μ in diameter. The nucleolus is small and stains deeply, while the reticulum is exceedingly delicate and difficult to distinguish (Figs. 7-9). The cytoplasm is very conspicuous owing to the large number of vacuoles. The most characteristic feature of the resting cell is the constant presence, in close contact with the nucleus, of a darkly stained mass of dense cytoplasm (Figs. 7-9), which is more or less definite in outline, although varying slightly in size and shape from cell to cell. It is usually more or less polygonal, but in a few cases is nearly circular. Occasionally it appears as a dense, but narrow, ring around the nucleus. Careful examination under higher powers always reveals the presence in this mass of a darkly stained granule (Figs. 4, 7-9), in intimate contact with the nucleus, in fact it appears to be situated on the nuclear membrane itself.

Such a cytoplasmic mass in close proximity to the nucleus has already been recorded by Carter (1926) in *Ulva lactuca*, although she did not observe the granule here recorded. She regarded this mass merely as a localized accumulation of cytoplasm, since, as I have also found, it cannot be traced during the process of nuclear division. The presence of the deeply-staining granule and certain other features described below suggest that this body may be centrosomal in nature.

As mitosis commences, the nucleus enlarges until it is nearly two or three times its original size; simultaneously there is a slight enlargement of the nucleolus and a definite reticulum, bearing numerous chromatin granules at the angles of the network, appears (Figs. 10, 11). No spireme was observed. The chromatin granules gradually become fewer but increase in size (Fig. 12), owing to coalescence. Finally, about ten chromosomes lying free in the nuclear cavity are formed (Fig. 13). The chromosomes at this stage are small, either rod-shaped or rounded, and of slightly different sizes (Fig. 13). The nucleolus persists without evident change throughout prophase and remains more or less brightly stained (Figs. 10-13).

At metaphase the chromosomes become arranged on an equatorial plate (Fig. 14). During this and the following stages the chromosomes form a more or less coalesced mass, so that determination of their number and of their mode of splitting into daughter chromosomes is difficult. All chromosome counts were, therefore, made from the prophase stages. The profile view of the late prophase nucleus generally gave ten as the number, while in a polar or oblique view the number appeared to be slightly larger or smaller. Determination of the diploid number, as well as of the number of gemini formed during diakinesis, shows that the correct number is 10.

The spindle appears when the chromosomes become arranged on the



Figs. 7-22. Stages in somatic mitosis in the gametophyte. Figs. 7-9. Cells with resting nuclei, showing the dense protoplasmic body with the centrosomal granule on the nuclear membrane. Fig. 10. Early prophase; nucleus with a distinct reticulum. Figs. 11, 12. Organization of chromosomes. Fig. 13. Late prophase showing ten chromosomes and the nucleolus. Fig. 14. Metaphase with centrosome-like bodies at the poles of the spindle. Figs. 15, 16. Anaphase. Figs. 17, 18. Late anaphase, with remains of nucleolus in middle of spindle. Fig. 19. Telophase. Fig. 20. Daughter nuclei just organized; reappearance of the centrosomal granule on the nuclear membrane. Figs. 21, 22. Formation of septum. (All figures $\times 1,100$.)

equatorial plate, but its mode of origin could not be ascertained. About this time in some preparations a rather deeply stained, centrosome-like body could be observed at the two poles. The spindle-fibres appeared to converge towards these bodies (Fig. 14), which were seen only during the metaphase and early anaphase (Figs. 14–16). During these stages the nuclear figure is situated in an almost clear cavity surrounded by faint traces of a membrane (Figs. 14–16), but it is difficult to say whether this is the persisting nuclear membrane or a secondarily formed cavity.

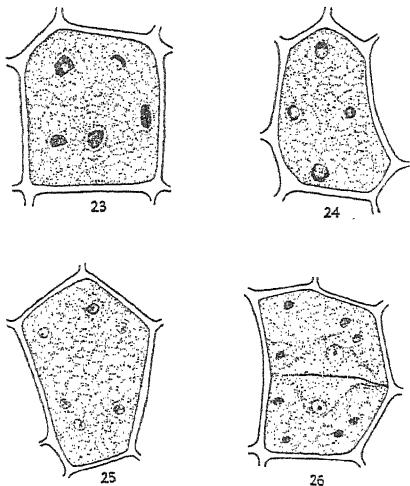
In the anaphase the nucleolus usually disappears (Figs. 15, 16), although in some instances a more or less deeply stained body, situated in the middle of the spindle (Figs. 17, 18), is evidently the persisting nucleolus. It is sometimes, however, rather difficult to distinguish this nucleolus from some of the chromosomes which often lag on their way to the poles. At telophase the chromosomes coalesce to form a compact mass and gradually lose their identity, becoming granular and taking up the stain less rapidly (Fig. 19). The two daughter nuclei are soon organized (Fig. 20). As the chromosome groups aggregate at the poles (Fig. 18), the spindle gradually fades away at the equator, but it remains indistinctly visible about the daughter nuclei, until a new nuclear membrane is formed. Each daughter nucleus shows a deeply staining granule on its nuclear membrane, like that recognized in the resting nucleus (Figs. 20–2), but the special cytoplasmic body associated with it is formed only later (Figs. 21, 22).

Whilst the two daughter nuclei are being organized, a delicate cleavage-furrow starts from the middle of that side of the cell near which the former are situated (Fig. 21) and extends gradually to the opposite side, where the chloroplast is usually located. In rare instances this cleavage-furrow appears while the nuclei are still in late anaphase. The plane of cleavage is usually through the middle of the cell (Figs. 21, 22), but is often slightly oblique, so that the two resulting cells are somewhat unequal in size. In still other cases the cleavage plane is more markedly oblique, extending to a lateral wall and giving two dissimilar cells. These less regular divisions give rise to cells arranged in no definite order.

The behaviour of the pyrenoid during vegetative nuclear division is interesting. In the cell, when not dividing, it shows a deeply stained 'pyrenocrystal' and an unstained starch-sheath surrounding it. As the nucleus passes to prophase, the pyrenoid gradually loses its starch-sheath and becomes progressively more lightly stained (Figs. 23, 24), until at metaphase it has completely lost its staining capacity and appears as a pale body, only to be distinguished with difficulty (Fig. 25). As the two daughter nuclei are organized, the pyrenoid gradually regains its staining capacity which is fully acquired when the two daughter cells are formed; the starch-sheath also soon reappears (Fig. 26). This suggests that some stainable substance stored in the pyrenoid is used up during the formation of the chromosomes and is gradually restored to it as nuclear division reaches completion. The pyrenoid does not really

disappear during division, but only loses a stainable substance, probably contained in an unstainable matrix, which is usually left behind (Fig. 25).

This behaviour of the pyrenoid is similar to that recorded by Carter (1926) in *Monostroma* and *Ulva*; she also describes, however, both a solution and a



FIGS. 23-6. Behaviour of the pyrenoids during somatic division. Figs. 23, 24. Cells from a diploid plant showing gradual loss of staining capacity of the pyrenoid body during prophase. Fig. 25. Cell showing condition of the pyrenoid body at metaphase. Fig. 26. Re-acquisition of staining capacity of pyrenoid as the daughter cells are formed. (All figures $\times 1,700$.)

stained nucleolus and a very faint reticulum; there is also the same cytoplasmic mass with an adjacent granule on the nuclear membrane.

In early prophase the nucleus increases to three or four times its original size (Fig. 27). The chromatin network becomes prominent and shows deeply staining chromatin granules at the angles. These granules become gradually fewer and larger until in late prophase ten chromosomes, varying slightly in shape and size, are formed; some are long rods, while others are small and rounded (Fig. 28).

At metaphase the chromosomes (Fig. 29) are again densely aggregated, but in a few clear instances the polar view at metaphase indicated ten chromosomes (Fig. 30). The spindle makes its appearance as the chromosomes become arranged on the equatorial plate (Fig. 29), and here also a deeply stained, centrosome-like body is found at each pole of the spindle during metaphase and anaphase (Fig. 29).

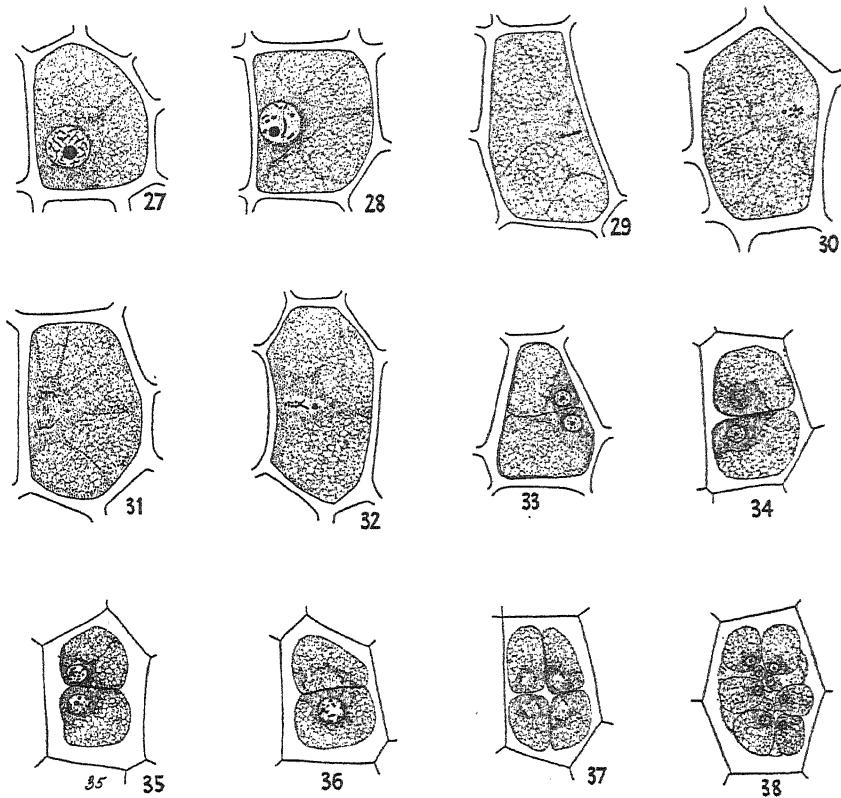
The nucleolus persists till metaphase, but usually disappears during ana-

fragmentation with dispersion of the fragments throughout the chloroplast and suggests that both these processes may go on at the same time. In the present instance, however, no fragmentation of the pyrenoid has been observed.

(b) Gametogenesis.

No difference in size or structure was found between the nuclei of male and female plants and in both the nuclear divisions were essentially the same. Any cell of the swarmer-producing portion can give rise to gametes by division of its protoplast, and before gamete-formation the cells in question are in no way distinguishable from those of the rest of the frond. As the period of gamete-formation approaches, the cells show a greater accumulation of contents and become more deeply stained. The nucleus is small, measuring about 2μ in diameter and, as in the vegetative cell, contains a deeply-

phase. In several instances, however, a rather deeply stained rounded body, generally located outside the spindle and much resembling the nucleolus, was seen both during metaphase and anaphase (Figs. 30-32). It looks as though the nucleolus had been pushed out into the cytoplasm, where it persists for



FIGS. 27-38. Stages in nuclear division during gamete-formation. Fig. 27. Early prophase. Fig. 28. Late prophase with ten chromosomes and the nucleolus. Fig. 29. Metaphase with centrosome-like bodies at poles of spindle and the persisting nucleolus. Fig. 30. Metaphase, polar view. Fig. 31. Anaphase, with nucleolus just outside the spindle. Fig. 32. Anaphase, polar view. Fig. 33. Formation of daughter nuclei. Figs. 34, 35. Daughter cells showing early (34) and late (35) prophase of the second division. Fig. 36. Second division in gametogenesis, showing the two nuclei in different stages of division. Figs. 37, 38. Formation of gamete-primordia. (All figs. $\times 1,200$.)

some time, but ultimately disappears. Such a behaviour of the nucleolus has been recorded by Wolfe (1904) and Cleland (1919) in *Nemalion multifidum* Ag. and by Maguitt (1925) in *Penium*, while among fungi it seems to be common. Derman (1933) records a similar expulsion of the nucleolus into the cytoplasm in *Callisia* and *Yucca*, for example.

Cleavage of the protoplast ensues while the daughter nuclei are organized

(Fig. 33). After a very short rest the daughter nuclei pass to the prophase of the second division (Fig. 34), the mitotic changes usually taking place simultaneously in both (Figs. 34, 35). During the second and subsequent divisions the nuclear figures are small and determination of the chromosome number is difficult, but in a few favourable preparations ten chromosomes were recognized during the prophase of the second division (Fig. 35).

Cleavage of the protoplast takes place more or less simultaneously, so that at the end of each nuclear division uninucleate somewhat angular primordia become constituted (Figs. 34–8). When division is complete the several primordia round off and assume the characteristic elongated shape of the gametes. Sixteen to thirty-two gamete-primordia are formed in each cell. During cleavage the protoplasm contracts slightly away from the wall so that the final cleavage products are crowded together towards the centre (Fig. 38). This contraction of the protoplast becomes evident, in most instances, after the first division is over (Fig. 33).

When fully formed the gametes escape through a rounded opening in the centre of the outer wall and apparently occupy the summit of a small beak-like projection. The gametes escape one by one, with the posterior end foremost.

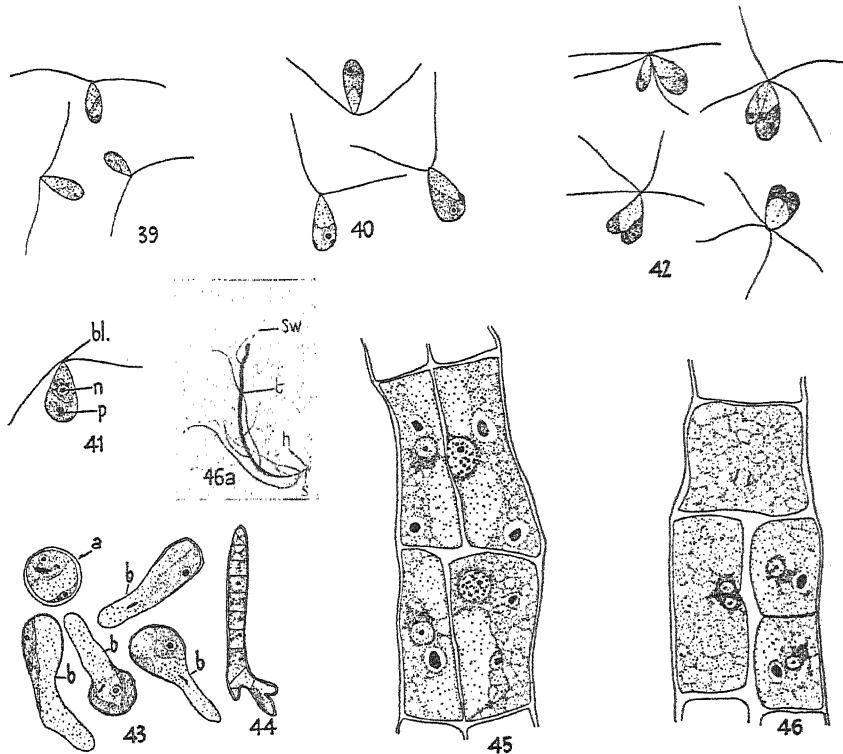
The early behaviour of the pyrenoid during gamete-formation is essentially the same as during somatic division, but here it becomes gradually altogether indistinguishable. It is probable that the unstained matrix divides continuously during gamete-formation, each primordium receiving a single piece of the pyrenoid matrix. These pieces evidently regain their staining capacity and become organized as the pyrenoids of the gametes.

(c) *Conjugation of the gametes and development of the zygote.*

A complete discharge of the gametes takes place in nature between 6 and 8 a.m., but in plants collected in the afternoon and left in the laboratory overnight the discharge takes place irregularly throughout the day until the evening. If, however, during any part of the day the water in which the plants have been standing is changed, a complete discharge ensues within a minute or two. It is thus possible to obtain gametes at any time of the day. As soon as liberated the gametes swim actively and swarm towards the illuminated side of the dish, where they continue to move for a period from a few to nearly twelve hours.

The more or less fusiform gametes have two long flagella attached anteriorly and somewhat subterminally (Figs. 39–41). At the pointed apex a distinct blepharoplast is always recognizable. The chloroplast with a single pyrenoid occupies usually one-third of the posterior region. The linear eye-spot lies laterally at the posterior end and the single nucleus is placed more or less centrally in the hyaline anterior portion (Fig. 41). The plants of *E. compressa* are, as already mentioned, dioecious. The conjugating gametes vary considerably in size (see p. 380), and all stages from pure isogamy to distinct anisogamy can be found (Fig. 42).

When a drop of water containing gametes from one plant is mixed with a drop containing gametes from another plant of opposite sex, there is a scramble for partners and numerous dense groups of gametes are soon formed. This kind of grouping was seen in all cases no matter what the proportion between



FIGS. 39-46a. Conjugation of gametes and development of zygote. Fig. 39. Male gametes. Fig. 40. Female gametes. Fig. 41. Female gamete, fixed and stained with iron-alum haematoxylin, showing nucleus (*n*) and blepharoplast (*bl*). Fig. 42. Conjugation of gametes. Fig. 43. Zygote (*a*) and stages in its germination (*b*); germinating zygotes in (*b*) 48 hours old. Fig. 44. Zygotic germling, 5 days old. Fig. 45. Cells from a zygotic germling showing stages in nuclear division; about twenty chromosomes can be counted in late prophase. Fig. 46. Another part of the same germling showing later stages in nuclear division. Fig. 46a. Zygotic germling, $1\frac{1}{2}$ months old. Note the small attaching disc (*h*), the short stalk (*s*), the intercalary growing portion (*t*) and the terminal empty swarmer-producing region (*sw*). (Fig. 46a about one-third natural size; Fig. 44 $\times 235$; other Figs. $\times 1,150$.)

the number of male and female gametes; it has been recorded by Hartmann (1929) and Bliding (1933) in *Enteromorpha* and by Föyn (1929, 1934b) in *Ulva lactuca*, although Miyake and Kuneida (1931) did not observe it.

The groups last only for a short time (from a few seconds to a minute) and soon the gametes separate in pairs, attached to one another by their anterior ends. Immediately after conjugation the paired gametes become negatively

phototactic and move towards the less strongly illuminated side of the drop. The actual fusion progresses gradually (Fig. 42) and occupies from a few seconds to several minutes, sometimes nearly a quarter of an hour. The fusing, as well as the fused gametes, usually remain motile for a short time. They finally attach themselves by their anterior ends, lose their flagella, and gradually round off. At this stage the zygote shows clearly the two chloroplasts, the two pyrenoids, and the two eye-spots derived from the two gametes (Fig. 43, *a*). It finally surrounds itself with a delicate wall, usually within three or four hours.

The zygotes germinate without undergoing any resting period and may commence to develop even after twenty-four hours. They lose their spherical shape, and become somewhat ovate. After forty-eight hours almost all show a somewhat hyaline protuberance at one end (Fig. 43, *b*). On the third day most consist of two cells, the one presumably basal having very few contents, while the other has a well-developed chloroplast with a large pyrenoid. Further development takes place rapidly, plants which are six or seven days old consisting of fifteen to twenty cells (Fig. 44). During this time, the basal hyaline cell divides to form a small holdfast of three or four cells which subsequently elongate and divide further. Some of the cells put out short branches so that finally a small attaching disc is formed. In addition, the cells immediately above the holdfast put out downwardly growing prolongations which become septate and produce a number of branches (Figs. 1, 2). In this way a cluster of branches is formed, which help in strengthening the primary attaching disc. The cells of this holdfast each contain a chloroplast with one or two pyrenoids and a single nucleus (Figs. 3–6).

These germlings, grown in artificial culture solutions, grew to the size of plants in nature, but growth was very slow, the full dimensions being only attained in two to three months. They became fertile within a month of germination and formed for several days numerous swarmers, which were all quadriflagellate and germinated without fusing.

(d) *Cytology of the germlings from zygotes.*

The young plants formed from the zygotes showed abundant nuclear division, irrespective of the time of fixation, whether during the day or during the night. In germlings of a centimetre or so in length, however, the divisions were more frequent during the night. The nuclear divisions were quite similar to those found in adult plants collected in nature. Figs. 45, 46 represent various stages of the vegetative mitosis in these germlings. The resting nucleus is like that of the somatic nucleus of the gamete-producing plant (Figs. 4, 45). At late prophase one finds nearly twenty chromosome units, i.e. exactly double the number observed in the somatic nucleus of the gametophyte (Figs. 5, 45). The germlings of the zygotes are thus all diploid and show clearly that no reduction division has taken place during the germination

of the zygote. The first nuclear division in the germinating zygote was unfortunately not obtained.

(e) *Parthenogenesis.*

A number of gametes were observed to germinate parthenogenetically. Such gametes retain their motility for quite a long time, but finally settle down. They become surrounded by a thin wall and remain in that condition for nearly a week. By the beginning of the second week they show signs of germination, which takes place in the same way as in the zygotes. Such parthenogenetic plants did not form the first set of swarmers until nearly two and a half months had elapsed.

Both male and female gametes can germinate parthenogenetically, though female gametes in general appear to do so more readily than the male. This statement is based on the fact that on two occasions the gametes formed from certain plants collected in nature were very large and presumably female; most of them failed to conjugate, and germinated parthenogenetically. They grew quicker and better than the other smaller gametes which were presumably male.

When mature, the parthenogenetic plants produced only biflagellate swarmers, which proved to be gametes and in all cases retained the sex of the parent. Plants from female gametes produced only female gametes and those from male gametes produced only male gametes.

Cytological examination showed that these parthenogenetic plants were all haploid. Föyn (1934b) records that certain parthenogenetic plants of *Ulva lactuca* showed a mixture of larger and smaller cells, the larger being diploid and the smaller haploid; the larger cells produced zoospores with four flagella, while the smaller ones produced only gametes. Nothing of this kind was found during the present investigation.

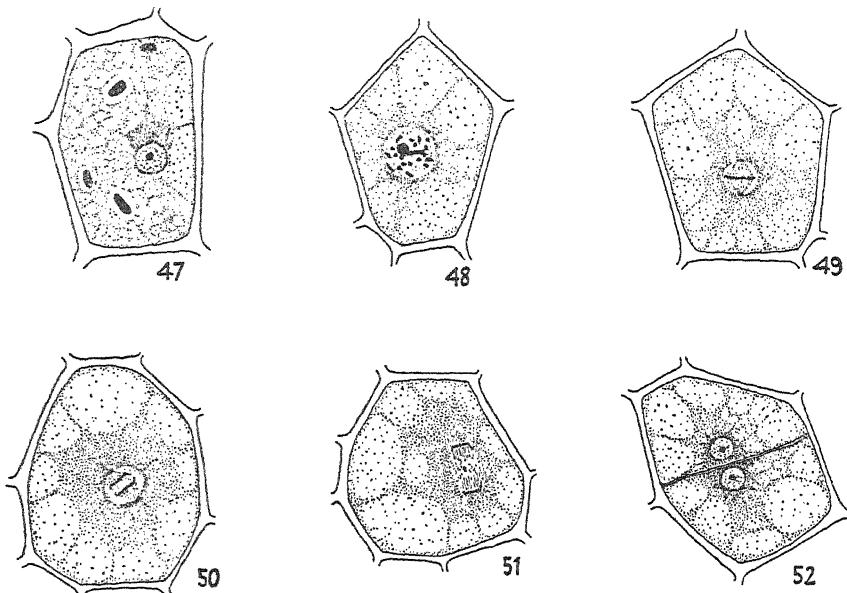
(f) *Somatic mitosis in the sporophyte.*

The cells of the thallus are in general similar in asexual and sexual plants. The resting, as well as the dividing nuclei, are, however, generally bigger in the asexual plant (cf. Figs. 7-22 with Figs. 47-52), being $3-4\mu$ in diameter as contrasted with the $2-3\mu$ of the gametophytic cells. Further, the number of pyrenoids in the chloroplast of the asexual plant is generally approximately double (3-5 or even 6) of that (2-3) of the sexual plant. The vegetative mitosis in the sporophytic cells resembles in all respects that in the gametophytic ones. Twenty chromosome units are organized in late prophase (Figs. 47-52).

(g) *Zoosporogenesis.*

The cells giving rise to zoospores differ only in their richer and denser contents. In early prophase the nucleus enlarges until it is three or four times the diameter of the resting nucleus and thus becomes very conspicuous

(Fig. 54). At the same time the reticulum becomes more prominent owing to the appearance of a number of darkly-stained chromatin knots at the junctions of the fibrils (Fig. 54). The amount of chromatin increases gradually as some of the fibrils break apart and disappear, while others thicken and become the more compact. This thickening and condensation continues until all the

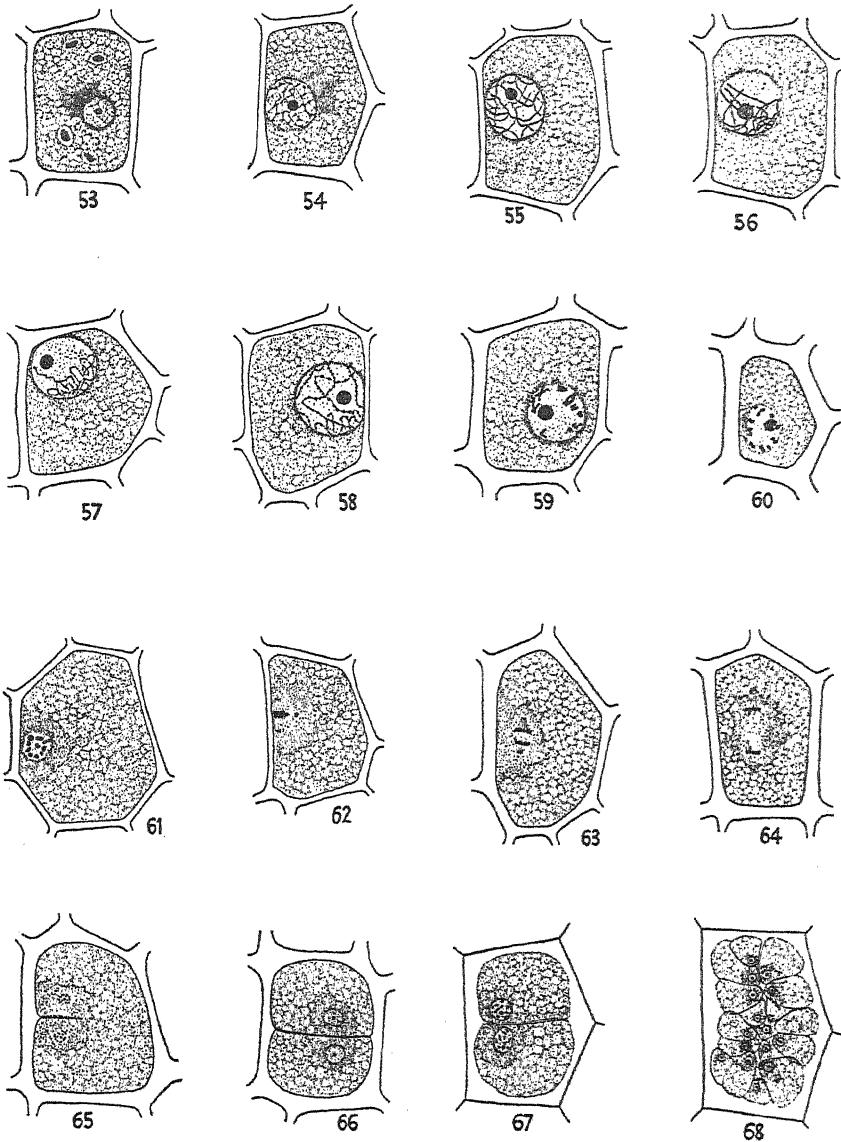


Figs. 47-52. Stages in somatic mitosis in the zoospore-producing plants. Fig. 47. Cell with resting nucleus. Fig. 48. Late prophase, with twenty chromosomes and the nucleolus. Fig. 49. Metaphase. Fig. 50. Mid-anaphase. Fig. 51. Late anaphase, with persisting nucleolus in middle of spindle. Fig. 52. Daughter cells fully formed. (All figs. $\times 1,300$.)

chromatic material becomes homogeneous in appearance and arranged in the form of a very distinct and much convoluted spireme (Fig. 55). This stage was never observed in mitotic divisions. The restriction of a spireme stage to the meiotic division is reported in *Codium tomentosum* (Williams, 1925), *Cladophora glomerata* (List, 1930), *Pylaiella litoralis* (Knight, 1923), and most Florideae (Westbrook, 1935).

Soon after the appearance of the spireme, the nucleus enlarges and the chromatin thread becomes more deeply stained and somewhat beaded (Figs. 56, 57). The thread then contracts and becomes gradually aggregated to one side of the nuclear cavity, where it appears as a dense mass of loops, leaving a clear central region (Figs. 56, 57). This phase of synezesis, which is of long duration, forms one of the most conspicuous stages of zoosporogenesis.

As the nucleus emerges from synezesis, loops arise from the knot and project freely into the nuclear cavity. In a few instances a number of loose ends



FIGS. 53-68. Stages in the reduction division during zoosporogenesis. Fig. 53. Cell with resting nucleus. Fig. 54. Early prophase. Fig. 55. Prophase, showing formation of spireme. Figs. 56, 57. Synkinesis. Fig. 58. Spireme emerging from synkinesis. Fig. 59. Early diakinesis, showing formation of ten bivalents. Fig. 60. Late diakinesis. Fig. 61. Metaphase of the first division; polar view, showing the ten gemini. Fig. 62. Early anaphase. Figs. 63, 64. Middle and late anaphase. Fig. 65. Telophase commencing cleavage. Fig. 66. Completion of cleavage. Fig. 67. Two daughter cells with nuclei in late prophase of the second division during zoosporogenesis, showing the ten chromosomes. Fig. 68. Completion of cleavage and formation of zoospore-primordia. (All figs. $\times 1,170$.)

were seen projecting from the knot and appeared to be extending into the cytoplasm (Fig. 56). In late prophase the entire spireme becomes redistributed throughout the nuclear cavity. Soon it begins to contract and to condense into short thick portions, finally giving rise to bivalent chromosomes (Fig. 59). The latter, though at first long and rod-shaped, gradually shorten and thicken and give rise to a distinct diakinesis phase which is, however, of very short duration (Fig. 60). The number of bivalents is always ten. The nucleolus remains practically unchanged throughout (Figs. 54–60).

After the condensation of the chromosomes the nuclear membrane gradually disappears and the bivalents become arranged in an equatorial plate (Fig. 61). Their grouping finally becomes so dense that their individuality becomes obscured. Only in a few polar views of metaphase could they be distinguished and the ten bivalents counted (Fig. 61). The spindle appears as the chromosomes become arranged on the equatorial plate. In side view of anaphase the achromatic figure is clearly seen with a centrosome-like body at each pole of the spindle (Fig. 62). Sometimes there is also evident a deeply stained, rather rounded body at one side (Fig. 62), representing the persisting nucleolus which again looks as though it had been pushed out into the cytoplasm (see p. 385).

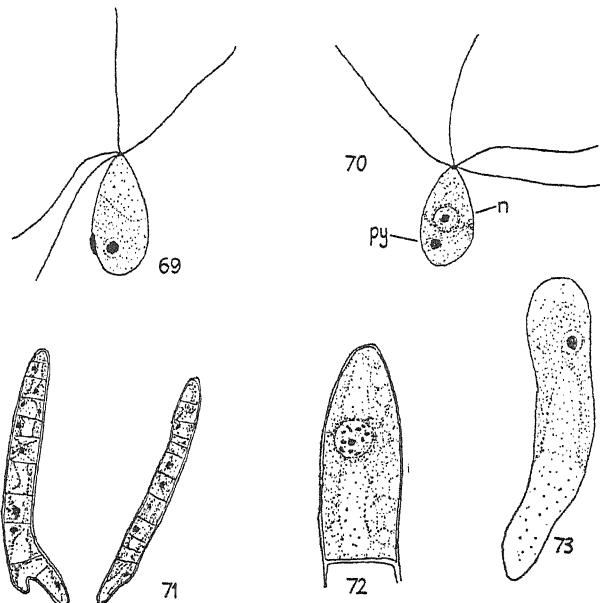
Anaphase and telophase follow rapidly upon one another, and the two daughter nuclei are soon organized (Figs. 63–6). Though the period of interkinesis is very short, each nucleus organizes a nucleolus and a faint reticulum; rarely a deeply-stained granule is seen on the nuclear membrane. Cleavage of the protoplast is evident before the second division commences (Fig. 66). The division of the two nuclei is in most cases simultaneous though occasionally successive (Fig. 67). The homotype presents no special features, except that ten univalents (the reduced number) are recognized in the prophase (Fig. 67). Further divisions follow in quick succession and usually occur simultaneously in all the nuclei. The cleavage of the protoplast again takes place in the main simultaneously with the nuclear divisions and a number of uninucleate zoospore-primordia soon become evident (Fig. 68). These primordia, at first angular, become rounded off only when cleavage is complete. The mature zoospores escape through a round hole in the centre of the outer wall of the cell, with their posterior end forward.

(h) The zoospores and their development.

The zoospores are discharged in the morning between 6 and 8 a.m., but they can be obtained at any time of the day by collecting material in the late afternoon and changing the water when the zoospores are required next day.

The zoospores exhibit positive phototaxy and retain their positive reaction for a few minutes or sometimes for one or two hours. Finally they become negatively phototactic and settle down on the darker side where they become attached by their anterior ends. The flagella are lost and the cell rounds off and becomes clothed with a delicate wall.

The zoospores are pear-shaped or occasionally fusiform (Figs. 69, 70) and are always larger than the gametes ($9\cdot8-11\cdot7\mu$ long, $4\cdot9-5\cdot9\mu$ broad). Each possesses four equal flagella which are attached subterminally and are longer than the body, a well-developed chloroplast with a large pyrenoid and a bright somewhat elongate eye-spot at the posterior end. The single nucleus is placed



Figs. 69-73. The zoospore and its development. Fig. 69. Zoospore. Fig. 70. Zoospore fixed and stained in iron-alum haematoxylin, showing nucleus (*n*) and the pyrenoid (*py*). Fig. 71. Germlings from zoospores 5 days old. Fig. 72. Terminal cell of a germling, showing the nucleus in late prophase with ten chromosomes. Fig. 73. Germling from zoospore, 48 hours old. (Fig. 71 $\times 325$; the rest $\times 1,600$.)

near the centre, just above the chloroplast (Fig. 70), while a distinct blepharoplast was always recognizable at the anterior end (Figs. 69, 70).

When placed in artificial culture solutions, germination of the zoospores commences almost at once and within twenty-four hours germ-tubes are formed (Fig. 73); within forty-eight hours the germlings become two-celled. Growth is very rapid and a holdfast is soon developed. Such germlings, like those from zygotes, become mature within a month of germination, when they produce biflagellate gametes; after a considerable time (sometimes 2-3 months) they grow to the size of the plants found in the sea. In nature, however, growth is usually much more rapid. The abundant nuclear divisions shown by the germlings are quite similar in all respects to those seen in the adult gametophyte (Fig. 72); in prophase ten chromosomes can be counted.

GENERAL DISCUSSION

The facts detailed above show that there are two kinds of plants, the one producing biflagellate gametes, the other quadriflagellate zoospores. Some of the sexual plants produce smaller gametes which may be regarded as male, while others produce larger gametes which may be considered to be female. Both possess ten chromosomes. The gametes formed by the two kinds of plants fuse with each other. The zygotes germinate without a rest period and grow into plants, whose cells have twenty chromosomes and which, when fully grown, produce only zoospores. During the formation of the zoospores reduction division takes place. The zoospores, after swarming for a while, settle down and grow into new plants, the cells of which possess ten chromosomes and which, when fully grown, produce only gametes, either male or female.

There is thus a regular alternation of haploid sexual and diploid asexual generations in the life-cycle, quite similar in all respects to that recorded by Föyn (1934b) in *Ulva lactuca*. The results of the present cytological study, further, fully confirm the conclusions of Hartmann (1929) and Bliding (1933) regarding the occurrence of alternation in Enteromorpha.

The structure and behaviour of the pyrenoids are quite similar to that found in *Ulva* and *Monostroma* as recorded by Carter (1926). The number of pyrenoids in the haploid cells is generally 2–3, while in the diploid cells it is 3–5. The larger number of pyrenoids in the diploid cells suggests that there may be a relation between the number of pyrenoids and the total chromatin content of the nucleus.

In the earliest stages of all mitoses studied, the nucleolus is brightly stained and very conspicuous. During the division stages it remains more or less unchanged and seems to take no active part, either in the formation of chromosomes or of the spindle. A noteworthy feature is its frequent persistence throughout nuclear division. In most instances it disappears completely at metaphase, but in a number of mitotic figures, both in the vegetative and reproductive cells, a rather deeply stained body, much resembling the nucleolus, was noticed during metaphase and anaphase. In the somatic mitoses this body was often seen in the middle of the spindle (Figs. 17, 18, 51), while in the gametangial divisions and during the first meiotic division it lay outside the chromosome-group (Figs. 29, 32, 62).

Every resting cell shows a densely stained protoplasmic body in close proximity to the nucleus (Figs. 7–9, 45, 47, 53), and in association with this body and situated almost on the nuclear membrane, a deeply stained granule. The latter disappears when nuclear division commences, but is seen again as soon as the daughter nuclei are organized (Figs. 20, 46, 52). During metaphase and anaphase, however, two deeply stained bodies make their appearance, one at each pole of the spindle (Figs. 6, 14–16, 29, 49, 50, 62, 63), although they disappear in early telophase. It seems probable that the bodies

at the poles of the spindle are formed by the division of the original single granule, although such division was not actually seen. It is suggested that the single granule on the nuclear membrane is centrosomal in nature and gives rise to the two centrosomes found at the poles of the spindle. The protoplasmic body, usually closely associated with the granule, may be regarded as a rudimentary centrosphere.

There has long been much uncertainty regarding the affinities and exact systematic position of the Ulvaceae. Bohlin (1901), Collins (1909), and Oltmans (1922) include it in the Ulotrichales, while Blackman and Tansley (1902), G. S. West (1916), and Smith (1933) place it as a separate order of the same status as the Ulotrichales; the last places it close to Schizogoniales. Wille (1909) and Printz (1927) rank it equal with Ulotrichaceae and Chaetophoraceae in the class Chaetophorales. Fritsch (West and Fritsch, 1927) separates the Chaetophorales as a distinct group from the Ulotrichales, and places the Ulvaceae as a separate family of Ulotrichales of equal rank with the Ulotrichaceae. In 1935 he ranks the Ulvaceae as a sub-section of Ulotrichineae in the order Ulotrichales.

The holdfasts of the germlings of *Enteromorpha* develop many horizontal and downwardly growing branches and the fully developed structure shows a great similarity to the prostrate system of some of the Chaetophoraceae (Fig. 74). In fact the developmental stages of the germlings closely resemble those of the germlings of some Chaetophoraceae, especially *Stigeoclonium* (see Fritsch, 1903, Figs. 19 and 21).

Fritsch (1927, p. 10) states that in the Chaetophorales there are (1) 'A prostrate system of creeping threads serving *inter alia* for attachment to the substratum' and (2) 'a projecting system more or less richly branched' (cf. also Fritsch, 1929, p. 111). He also points out that two lines of development are seen within the Chaetophorales. In the one there is a gradual reduction of the projecting and a corresponding increase of the prostrate system until finally the projecting system is completely eliminated. In the other there is a gradual increase of the projecting system and a reduction of the prostrate system. In extreme cases, as in *Draparnaldia*, the prostrate system is reduced to a minimum. The germlings of *Enteromorpha* show both these systems, viz.

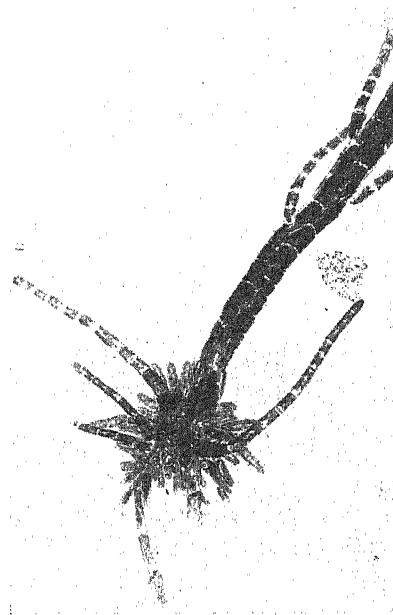


FIG. 74. Basal region of a zoosporic germling, 20 days old, showing the holdfast and a number of proliferations arising from it. ($\times 630$.)

(1) an erect branched filamentous portion, and (2) a prostrate, much branched rhizoidal portion. Thus, a consideration of the early stages of Enteromorpha suggests that the Ulvaceae might have been derived from some Chaetophoraceous ancestor, although this affinity has become obscured in the mature plant owing to the early longitudinal division of the cells and the consequent elaboration of the thallus into a parenchymatous structure.

Longitudinal division of the cells of the thallus is not unknown in the Chaetophoraceae, having been recorded in *Stigeoclonium variable* Nág. (Gay 1891, Pl. VIII, Figs. 68–72), in young filaments of *Draparnaldia glomerata* (Gay, 1891, Pl. X, Fig. 95), and in *Fritschella* (Iyengar, 1932, Fig. 2). A consideration of these facts suggests that Enteromorpha may have evolved from a Chaetophoraceous ancestor by reduction of the prostrate system into a compact attaching disc and by elaboration of the projecting system into a thalloid parenchymatous structure. The profuse branching, found in certain forms of Enteromorpha, may also be related to the tendency of the projecting system of Chaetophoraceae to branch.

This hypothesis is open to the objection that the branches formed by the holdfast may be abnormalities due to cultural conditions. Practically all the plants cultivated during the present investigation, however, showed this type of branching of the holdfast and no abnormal features were detected in the upper regions of the plants. Some of the figures of Bliding (1933, Figs. 2b, 9e, and 12e) suggest that a similar tendency to form a prostrate system is present in other species of Enteromorpha also.

SUMMARY

1. An account is given of the structure of the thallus of *Enteromorpha compressa* (L.) Grev. var. *lingulata* (J. Ag.) Hauck. The structure and development of the holdfast is described in detail.

2. The life-history, as followed in the laboratory by means of cultures, is described.

3. A cytological study of the alga collected in nature shows that the sexual plants have ten and the asexual twenty chromosomes. The gametophyte is dioecious. Sexual fusion shows all stages, from pure isogamy to definite anisogamy.

4. The zygotes germinate without a resting period and the germlings are diploid with twenty chromosomes. The mature plants grown from zygotes produce only quadriflagellate zoospores.

5. Reduction takes place during the formation of zoospores. A distinct spireme, a synezetic knot, and a typical diakinesis form the conspicuous features of this division. Ten bivalent chromosomes were observed at the first division and ten univalent chromosomes in the subsequent ones.

6. The zoospores develop immediately into haploid germlings with ten chromosomes. The resulting plants produce only gametes.

7. There is thus a regular alternation between a haploid gametophyte and a diploid sporophyte, the two generations being externally altogether alike. The number of pyrenoids in diploid cells is nearly double that in haploid cells. A possible relation is suggested between the number of pyrenoids and the chromatin content of the nucleus.

8. Parthenogenetic development of the gametes has been observed. The resulting plants were all haploid and produced only gametes.

9. The behaviour of the nucleolus during nuclear division suggests that it plays no active part either in the formation of the chromosomes or of the spindle, but that it is often pushed out into the cytoplasm.

10. A deeply stained body, probably of the nature of a centrosome, is always discernible on the membrane of the resting nucleus.

11. The systematic position of the Ulvaceae is discussed and a possible relation to the Chaetophoraceae suggested.

In conclusion, the author desires to express his great indebtedness to Professor Iyengar, Director of the University Botany Laboratory, Madras, for suggesting the problem and for constant guidance and helpful criticism throughout the course of the work. His thanks are also due to the authorities of the University of Madras for the award of a research scholarship, during the tenure of which the present investigation was carried out. He is also indebted to Professor Fritsch for assistance in preparing the manuscript for press. This paper formed part of a thesis submitted for the M.Sc. Degree of the University of Madras.

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A Study of Australian 'Sooty Moulds'

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With Plate XII and four Figures in the Text

I. TAXONOMY

Introduction

THE dark coverings of fungal growth which occur on the leaves and stems of various plants have long been recognized as 'sooty moulds', but the taxonomy of the individual components of these coverings is still very confused. One of the objects of the present paper is to analyse critically existing schemes for the classification of 'sooty mould' fungi, and to suggest one which is consistent with recent research.

Review of Recent Literature

A taxonomic study of the 'sooty moulds' may be approached conveniently by consideration of Arnaud's investigations. After conducting an extensive examination of the 'fumagines' occurring in the vicinity of Montpellier (1910a, b), he (1911, 1912) made a survey of the fumagoid species listed by Saccardo, and he suggested some important emendations to the method of classification adopted by that author.

Saccardo (1883) referred 'sooty mould' fungi possessing flat, shield-like perithecia to the Microthyriaceae Sacc., but all the other species he retained in the Perisporiaceae Fr. The latter family he distinguished from the Sphaeriaceae Fr. merely by the non-ostiolate character of its perithecia (Sacc., 1882).

This distinction was not accepted by Arnaud (1911), who completely abolished the Perisporiaceae. Later (1918), although he retained the saprophytic 'fumagines' in the Sphaeriaceae Arnaud, recognizing the parasitic genera to be distinct, he transferred some to the Microthyriales auct. emend. and others to the Dothideales Th. & Syd. emend.

Although Gäumann (1928) adopted Arnaud's system of classification in its essentials, he did not entirely abolish the Perisporiaceae; but he retained that family for the inclusion of Meliola and similar ectoparasitic genera.

Von Höhnel did not examine the 'sooty moulds' particularly, but his study

of fungi generally included a number of species belonging to this group, and he made some valuable suggestions concerning their classification.

He (1909b, p. 1198) directed attention to the fact that an unreliable character, the vertical elongation of the perithecia had been used by Saccardo for the separation of the tribe Capnodieae Sacc. from other members of the Perisporiaceae Fr. He suggested that the following features of the perithecium would be taxonomically more useful: (i) the tough and mucilaginous texture, (ii) the non-ostiolate character. Later (1918) he assigned the Capnodieae to the Pseudosphaeriaceae. The latter family was created by von Höhnel (1907a) to include fungi which he regarded as transitional between the Dothideaceae aut. and Sphaeriaceae aut.

Genera such as *Limacinula* were distinguished from the Capnodieae by the membranous, soft texture and ostiolate structure of their ascocarps; and von Höhnel created a new section of the Sphaeriaceae to accommodate them. Originally this section was named *Naetrocymbeae* (1909b), but later (1918), for reasons of priority in nomenclature, Coccodineae was substituted.

The position of parasitic genera, such as *Meliola* and *Asterina*, was not clearly indicated in his scheme of classification.

Theissen and Sydow (1917) gave a detailed classification of a large number of Ascomycetes, including the 'sooty mould'-fungi. The family Microthyriaceae, characterized by flat, shield-shaped ascocarps, was referred to the order Hemisphaeraiales; but all the other 'sooty mould'-genera were included in the Perisporiales Lindau. This collection of genera they assigned to the Perisporiaceae and Capnodiaceae, the second family being distinguished by the mucilaginous nature of the mycelium and perithecia.

These authors did not subscribe to von Höhnel's view concerning the segregation of the Coccodineae from the Capnodieae, as they did not consider a perithecial ostiole to be a distinctive character. The Capnodiaceae v. Höhn. was accepted as a family comprising species with ostiolate as well as non-ostiolate perithecia; in other words, it was regarded as a connecting link between the typically cleistocarpous Perisporiales and the ostiolate Sphaerales. The Pseudosphaeriaceae v. Höhn. they (1918) recognized and raised to ordinal rank; but unlike von Höhnel, they did not include in it the genera of the Capnodieae.

It is apparent that Theissen and Sydow did recognize some natural cleavage in the large assemblage of genera which they assigned to the Capnodiaceae; and the family was divided into two sections which were characterized by stalked and sessile perithecia respectively. The genera referred to the Coccodineae by von Höhnel were included in the second group by these authors.

Woronichin (1925) directed attention to the fundamental nature of the distinction between the Capnodieae and the Coccodineae, but he did not agree with von Höhnel's taxonomic treatment of them. Like Theissen and Sydow, he considered that the Coccodineae v. Höhn. was very closely related to the Capnodiaceae. He therefore suggested the formation of a new order,

the Capnodiales, in which are included both the Capnodiaceae v. Höhn. and the Coccodiniaceae v. Höhn. This order, from perithecial structure, may be regarded as a link between the Perisporiales and the Sphaeriales.

There are certain cytological investigations which are relevant to the study of 'sooty mould' taxonomy.

The family Pseudosphaeriaceae was formulated by von Höhnel (1907a) to accommodate species with fructifications, which, though resembling true perithecia, are in reality of stromatic nature. The family was recognized by Theissen and Sydow (1918) and Petrak (1923); but these investigators were not unanimous concerning its limitations.

It is Miller's view (1928a) that this disagreement arose from the failure to differentiate clearly between the perithecial wall and the stroma. Tissue enclosing the ascigerous centrum was regarded by Miller (p. 194) as a perithecial wall only if it arose from the archicarp. Stromatic tissue was defined as 'coalesced hyphae, which do not arise as a result of a sexual stimulus' (p. 191). This interpretation of the perithecial wall makes the line of demarcation between the orders Sphaeriales and Dothideales quite clear, and Miller included the genera formerly assigned to the Pseudosphaeriales in the Dothideales.

Fraser (1935a) made a cytological study of some 'sooty mould' fungi, and she interpreted her observations according to Miller's conception of perithecial wall formation. Upon this evidence, she referred the Capnodiaceae to the Dothideales.

Discussion and Criticism of Recent Investigations

Arnaud.

The distinction between parasitic and saprophytic 'sooty moulds', which was made almost simultaneously by Arnaud (1918) and Neger (1918), appears to be valid. Some are ectoparasitic, deriving nutriment from the host either by means of haustoria or by chemical corrosion of the leaf-cuticle. These species are apparently confined to the families Microthyriaceae and Perisporiaceae. In other circumstances the host constitutes an inert substratum, and the 'sooty mould' grows saprophytically in the 'honey dew', which covers the leaves and stems of plants attacked by scale-insects and aphids.

There are other features of Arnaud's classification which are, however, not so satisfactory. In the first place, the assignment of the saprophytic 'fumigines' to the Sphaeriales was based upon a very indefinite interpretation of the term 'ostiole'. Arnaud extended that term to include any region of the perithecial wall which is thinner than the rest, even though this character may only be detected by sectioning.

Secondly, Arnaud's treatment of the universally recognized genus *Capnodium* Mont. constituted a rather revolutionary step. He abolished this genus, because he considered that Montagne's description was based upon pycnidial and not perithecial characters. The fact is, however, that although Montagne

did not discriminate between pycnidia and perithecia some fructifications of the latter type were seen, and asci were described by him (1849a). Thus the generic name *Capnodium* was applied by Montagne to the ascigerous stage, and it is therefore quite valid.¹

Thirdly, Arnaud's conception of genera is too comprehensive. This criticism may be well supported by reference to *Limacinia Neger*. The genus had already been given a very broad interpretation by Saccardo; in vol. xiv of the 'Sylloge' it was included in the tribe *Capnodieae*, for the reception of all species with sessile perithecia. Consideration was not given to other widely divergent characteristics such as smooth or setulose perithecia and dark or colourless, muriform or phragmo-septate, spores. In vol. xvii of the 'Sylloge', however, species with spores muriformly septate were separated into the subgenus *Limacinula* Sacc.

Arnaud regarded perithecial shape as an unreliable taxonomic feature, and he (1911) did not limit the genus *Limacinia* to species with sessile perithecia. The generic character that he insisted upon was that the spores should be phragmoseptate, irrespective of their colour.

Von Höhnel (1910a) and Theissen and Sydow (1917) agreed with Arnaud, in so far as they included in this genus only species with phragmoseptate spores. However, while on the one hand von Höhnel's key insists on brown spores, that of Theissen and Sydow just as definitely attributes colourless spores to the genus *Limacinia*. The latter authors further limited the genus to species with smooth and sessile perithecia.

The existence of such varied interpretations of the genus *Limacinia* suggested that the time was ripe for a re-examination of the type-specimen and for a clearer definition of the genus. The type-specimen of *Limacinia* proved to be unprocurable, although application was made to several institutions in which Johow's specimens are preserved, and so reference was made to the original description (see Johow, 1896). In this the mature spores are clearly stated to be dark and phragmoseptate, but colourless immature spores are also described. In this way, doubtless, the confusion concerning the colour of the spores has arisen. The perithecial characters are given as spherical and non-ostiolate; no mention is made of bristles. Thus, if the classification suggested by Arnaud is to comply with Neger's description, all species with colourless spores should be excluded from the genus *Limacinia*. Some of these species may be transferred to the genus *Scorias* Fr.

The latter was regarded by Arnaud merely as an aggregation of *Limacinia* spp. exhibiting an abundant formation of mucilage; it should, however, be retained as a separate genus. Although, when *Scorias* was first described in

¹ In earlier publications by the writer (1933, 1935) Arnaud's system of nomenclature was followed; two generic names which appear in these are now rescinded. Firstly, *Capnodium* should be used instead of *Teichospora* Fuck. The latter genus was described more recently than *Capnodium*; moreover *Teichospora* may be distinguished by the fact that the mycelium is embedded in the substratum. Secondly, in accordance with the rule of priority, the form-genus *Chaetophoma* Cke. is replaced by *Antennularia* Reichb.

the ascigerous stage, Montagne (1849b) directed attention to its mucilaginous nature, he also mentioned the colourless and phragmoseptate spores as a generic character. Furthermore, the pleomorphic character of 'sooty mould' fungi has been disproved by subsequent investigators (Neger, Tengwall, Fisher, Fraser) who, by 'pure culture' methods, have clearly demonstrated the composite nature of these coverings.

Von Höhnel.

Unlike Arnaud, von Höhnel did not separate the parasitic from the saprophytic type of 'sooty mould'. von Höhnel's classification is, however, more satisfactory, for his generic conceptions are narrower. Furthermore, his interpretation of ostiolar structure required direct microscopic examination only; it was not dependent upon evidence derived from fructifications which had been sectioned.

Upon this basis, a non-ostiolate perithecium was attributed to the Capnodieae as a constant character. This was confirmed by the writer's study of *Capnodium* spp., *Scorias* spp., and *Limacinia* spp.

However, the mucilaginous texture, the other diagnostic feature which von Höhnel ascribed to the Capnodieae, does not commend itself so strongly. This appears to be a general characteristic of epiphytic fungi, e.g. *Phycopsis* sp., which taxonomically is very remotely related to the Capnodieae, is also extraordinarily mucilaginous. Nevertheless, of all the classificatory schemes which have been offered, that suggested by von Höhnel appears to be the most consistent with present views concerning the 'sooty mould' fungi.

Theissen and Sydow.

These authors (1917, pp. 471–3) formulated a key to the genera of the Capnodiaceae which, possibly because of its comprehensive character, has been used by many subsequent investigators for the identification of specimens.

This classification, however, was established upon certain inaccuracies. A misconception is perpetuated concerning the shape of the perithecia in the genus *Capnodium*. Using this false character as the criterion of segregation, the family was divided into two sections: (i) Eu-Capnodieae Th. and Syd., in which the perithecia are pedicellate, and (ii) Chaetothyrieae Theiss., with sessile perithecia. It is the writer's experience (1933, p. 182) that such a separation of genera is unworkable; a specimen of *Capnodium salicinum* with sessile perithecia would be identified as a species of *Coccodinium*.

Furthermore, in regard to the spore characters of the genus *Capnodium*, this classification is ambiguous. It appears from the definition of the genus, that Theissen and Sydow (p. 473) included in it phragmoseptate species such as *Capnodium Walteri* Sacc.; yet provision for *Capnodium* spp. has only been made in the muriform-spored section of the key.

The genus *Limacinia* has already been discussed in detail, and it has been noted that, according to Neger's original description, the mature ascospores

are dark in colour. Theissen and Sydow erroneously attributed colourless spores to *Limacinia*, and they created a new genus *Phragmocapnia* to include species with brown spores.

Fraser.

For the purpose of identifying the 'sooty moulds' of New South Wales Fraser used Theissen and Sydow's key, and the artificiality of the classification suggested by the latter authors may be illustrated by reference to her paper (1935b). By comparison of the ascocarps of *Capnodium Walteri* (Fraser, fig. 10) and *Henningomyces affine* (Fraser, fig. 71) no real difference is revealed, and yet according to the classification suggested by Theissen and Sydow these two genera represent the Eu-Capnodieae (ascocarps stalked) and Chaetothyrieae (ascocarps sessile) respectively. Moreover, the inclusion of *Henningomyces affine* and *Limacinia concinna* in a study which purports to deal with the species of the Eu-Capnodieae suggests that Fraser considered their affinities to be with this group rather than with the Chaetothyrieae, in which both genera were included by Theissen and Sydow. However, no explanation of the anomaly has been given by Fraser, although a paper dealing with the species of the Chaetothyrieae was published by her two months later.

Apart from the unreliability of the scheme of classification which Fraser followed, it would appear that she has been misled in the identification of the species described as *Aithaloderma viridis* n.sp. The ascocarp dimensions quoted by Fraser (1935a) indicate a flattened shape, and reference was made also to radiating cracks which appear in the wall of the ascocarp at maturity. In agreement with other authors, Theissen and Sydow (1917, p. 413) regarded fructifications of the type described above as characteristic of the family Microthyriaceae Sacc., and the identification of Fraser's specimen should, therefore, be sought amongst the genera of that family. Moreover, Fraser herself has stated that 'the young pycnidia resemble the fructifications of the Microthyriaceae'. It is the author's opinion that Fraser's fungus should be identified as a member of the Microthyriaceae. This has been confirmed by the examination of two specimens sent out by Dr. Fraser. One, which is illustrated in Pl. XII, Fig. 7, was forwarded directly to the writer, and the other to the Kew Herbarium.

Cytological Investigations

(a) *Capnodiaceae* v. Hohn. emend. E. Fisher and *Chaetothyriaceae* Th. emend. E. Fisher.

Fraser (1935a) studied the cytology of four fungi which she identified as follows: *Capnodium salicinum* Mont., *Capnodium salicinum* var. *uniseptum* n. var., *Aithaloderma ferruginea* n.sp., *Aithaloderma viridis* n.sp. Interpreting her observations in the light of Miller's investigations Fraser referred these to the Dothideales. She concluded that development is essentially the same in

all four fungi, and that the archicarp is a multicellular structure arising in a stroma. However, until the exact origin of the archicarp is determined, the possibility still exists that some of the tissue described by Fraser as stromatic may arise from the archicarp, and so constitute a true perithecial wall.

This criticism may be applied particularly to the species assigned by her to the genus *Aithaloderma*; the stromatal core of the fruit-body in both *A. ferruginea* and *A. viridis* was described as 'compact and wall-like'.

Fraser did not observe any differentiation of internal stroma in the ascocarp of *Capnodium*, and the assignment of this genus to the Dothideales may be accepted with more certainty.

(b) *Microthyriaceae* Sacc.

Although perithecial development in the *Microthyriaceae* has long been a subject of investigation, no detailed cytological knowledge of the family is available.

The examination of a large number of species by Ryan (1926) still left the subject obscure. It merely showed that the perithecia arose on the lower surface of the mycelium as flattened plates of cells, and no detail concerning archicarp or ascal formation was observed.

If the identification of *Aithaloderma viridis* L.Fr. as a member of the *Microthyriaceae* were accepted (see above) a more detailed cytological knowledge of the latter family would thereby be available. Fraser's study of ascocarp development in *A. viridis* and *A. ferruginea* revealed a certain similarity of structure, which suggests that the *Microthyriaceae* is closely related to the *Chaetothyrieae* Th.

(c) *Perisporiaceae* Fr.

Valuable information bearing on the cytology of the *Perisporiaceae* was supplied by Graff's observation of ascocarp development in *Meliola circinans*. In this species Graff (1932) described an antheridium and a uninucleate oogonium from the base of which hyphae developed to form a true perithecial wall.

Graff indicated the striking similarity between *Meliola circinans* and the *Erysiphaceae* in the matter of ascocarp development; but at the same time two features of the mature fructifications were noted, which appeared to be distinctive of the former species. In *M. circinans* an ostiole was observed, and the outermost layers of the ascocarp wall, which arise from vegetative hyphae, were described by Graff as stromatic. On the other hand, in the *Erysiphaceae* the ascocarp is typically non-ostiolate and its entire wall arises from the oogonial hypha. However, an investigation of additional species is required before the presence of an ostiole and a stroma can be regarded as characteristic of the *Perisporiaceae* in general.

Proposed Classification

It is evident from the preceding survey of literature that unanimity has not been achieved by those who have considered the classification of 'sooty mould' fungi. Each suggested scheme of classification has resulted from the recognition of some intrinsic attribute of the fungi concerned, but unfortunately at the same time false or uncertain characters have frequently been utilized.

The following classification is suggested after making a critical analysis of existing taxonomic schemes in the light of recent research:

A. Components not confined to a 'sooty mould' habitat.

These are ubiquitous saprophytes which usually grow on decaying organic matter, but which also grow abundantly in the 'honey dew' secreted by scale insects and aphids. They may be designated as unspecialized components.

Particular attention has not been paid to the taxonomy of these fungi. Some, such as *Dematium pullulans*, *Cladosporium* sp., and *Macrosporium* sp., belong to genera of the Fungi Imperfetti; but *Pleospora herbarum*, which has been referred to the much disputed Pseudosphaeriaceae v. Höhn., is also of common occurrence.

B. Components endemic to a 'sooty mould' habitat.

These grow only on the surface of living plants or on some 'honey dew'-covered substratum.

The diagnostic features of the families to which these species belong are summarized in the following key:¹

KEY TO FAMILIES

(a) *Intense black covering, spreading uniformly over the surface of the host, frequently forming a thick layer* (see Pl. XII, Figs. 1 and 2).

I. *Capnodiaceae* v. Höhn. emend. E. Fisher.

Vegetative hyphae free, conspicuously and very regularly septate. Cytological evidence suggests that the archicarp is multicellular; there is no differentiation of tissue immediately enclosing the ascigerous centrum.

Spherical ascocarps may be stalked or sessile.

Hypopodia and haustoria absent.

Growth on artificial culture-media is possible.

(b) *Sooty film always thin and membranous* (see Pl. XII, Figs. 4 and 5).

(i) Vegetative hyphae when present, are free, irregularly septate.

Cytological evidence suggests that the archicarp is multicellular; the tissue immediately surrounding the ascigerous centrum is differentiated from the rest of the enclosing tissue.

¹ As the writer is not familiar with the Trichothyriaceae Th. this family is omitted.

- (x) Ascocarp hemispherical and structure not radial (see Pl. XII, Fig. 3). II. Chaetothyriaceae Th. emend. E. Fisher.
Hyphopodia and haustoria absent.
Growth on artificial culture-media has been recorded for some species.
- (y) Ascocarp flat and structure radial (see Pl. XII, Fig. 7). III. Microthyriaceae Sacc.
Hyphopodia and haustoria present in some species.
Growth on artificial culture-media has been recorded for one species.
- (ii) Hyphae always united to form a membranous thallus. IV. Trichopeltaceae Th.
Cytology not investigated, but the asci arise within fertile areas of the thallus ('pyknothecien').
Growth on artificial culture-media has been recorded.
- (c) *Black growth, restricted to isolated areas of the host surface* (see Pl. XII, Fig. 6). V. Perisporiaceae Fr.
Vegetative hyphae free.
The available cytological evidence suggests a relationship with the Erysiphaceae. The archicarp is unicellular; and the tissue immediately enclosing the ascigerous centrum is developed from sexual hyphae.
Spherical ascocarps always sessile.
Hyphopodia and haustoria present.
Growth on artificial culture-media not recorded.
- (d) *Inconspicuous growth, covering the surface of the host with minute blackish-brown spots* (see Pl. XII, Fig. 8). VI. Atichiaceae Millardet emend. Rac.
Hyphae absent. Thallus consisting of chains of bead-like cells embedded in a mucilaginous matrix.
The cytology of these forms has not been investigated, but the asci arise within the tissue of the thallus.
Growth on artificial culture-media is possible.

In formulating this classification recognition has been made, as far as possible, of the development as well as the mature structure of the ascocarps. These are subject to modification by external conditions to a lesser degree than are vegetative characters.

Unfortunately, very little information of this kind is available for the 'sooty mould' fungi. The number of species which have been examined cytologically is very limited, and practically all of the observations are incomplete.

The necessity for accurate observation in connexion with ascocarp shape is demonstrated by the genus *Capnodium*, in which confusion ensued from

the perpetuation of an erroneous observation. However, if accurately described, the shape of the ascocarp may prove a valuable feature of distinction as, for example, between the Chaetothyriaceae and the Microthyriaceae. Another noteworthy feature of ascocarp structure is the ostiole, but the limits of that term require sharper definition before any taxonomic significance can be ascribed to it.

Immediate requirements make a key to the identification of these fungi desirable. It has been necessary, therefore, to supplement our knowledge of cytology and ascocarp structure with characters which are possibly less reliable, but which nevertheless appear to be diagnostic.

Field observations indicate that 'sooty moulds' may be separated into more or less distinct groups according to the colour and texture of their growth. In this way the endemic 'sooty mould' fungi are classified in four sections. One section has been further subdivided, so that six families are enumerated, each of which will now be considered in detail.

FAMILY I. *Capnodiaceae* v. Höhn. emend. E. Fisher.¹

A.	Spores muriform-septate and brown	. Capnodium Mont. syn. Capnodaria Sacc.
B.	Spores phragmoseptate	
(a)	Spores brown Limacinia Neg. syn. Perisporina P. Henn.
(b)	Spores hyaline Scorias Fr. syn. Paracapnodium Speg. Henningsomyces (Sacc.) emend. v. Höhn.
C.	Spores 2-celled Capnodiella Sacc.
D.	Spores 1-celled	

This scheme of generic separation is essentially that suggested by von Höhnel (1910a); certain genera, however, which that writer included in the Capnodieae are eliminated and others fall into synonymy.

Excluded genera.

The exclusion of the genera cited below is necessitated by the fact that von Höhnel did not discriminate between parasitic and saprophytic 'sooty moulds'; on the other hand, the family Capnodiaceae as defined in this paper comprises non-parasitic species only.

In *Kusanobotrys* P. Henn., *Alina* Rac., and *Balladyna* Rac. penetration of the host-tissue has been described. The exclusion of *Perisporiopsis* P. Henn. and *Dimerosporina* v. Höhn., however, is established upon the evidence of

¹ Except for those specified below, references to the original descriptions of the genera included in this family are contained in the text.

Henningsomyces Sacc. Syll. Fung., vol. xvii, Emendation by von Höhnel Sitzungsber. k. Akad. Wiss. Wien, math-naturw. Kl., Bd. 119, Abt. 1, 460.
Capnodiella Sacc. Syll. Fung., vol. i, 73.

the growth-habit of these fungi, which is very similar to that of the Perisporiaceae.

Synonymy.

One of the most valuable features of von Höhnel's system of classification is that his conception of genera is sharply defined. An exception is provided, however, by the genus *Capnodaria* Sacc. von Höhnel extended this genus, including in it both muriform and phragmosporous species. Originally the name *Capnodaria* was applied by Saccardo (1882, p. 74) to a sub-genus of *Capnodium* which was characterized by the development of sixteen spores in the ascus. Unlike von Höhnel, the writer does not consider the latter character to be worthy of generic distinction, and so *Capnodaria* Sacc. is listed as a synonym of *Capnodium* Mont.

Von Höhnel suggested that, because of the cylindrical shape of its spores, the genus *Perisporina* P. Henn. (1904) should be separated from *Limacinia* Neger. This distinction is not an adequate basis of generic separation, nor does it appear to be accurate. The type-material of *Limacinia* Neger was not available, consequently a comparison of actual specimens was impossible. However, the descriptions and figures given by the respective authors suggest that *Limacinia* Neger and *Perisporina* P. Henn. are identical, and upon this evidence the suggested synonymy is established.

The Capnodiaceae v. Höhn. was enlarged by Theissen and Sydow (1917), but most of the additional genera are extraneous according to the definition of the family given in this paper. Only *Paracapnodium* Speg. shows any affinity with the genera of the Capnodiaceae v. Höhn. emend., and this is included as a synonym of *Scorias* Fr. The type-material of *Paracapnodium* Speg. has not been examined, but, according to the description given by Spegazzini (1909, p. 325), this genus was only differentiated from *Scorias* Fr. by the presence of stellate conidia in the former genus. Such a separation is unsatisfactory as the conidial stage of *Paracapnodium* cannot be accepted as an authentic feature until it has been connected by 'pure culture' methods with the perfect stage.

The muriform-septate character of the spores, which was chosen by Theissen and Sydow (1917, p. 472) as the diagnostic feature of *Paracapnodium* Speg., is also unreliable. Although one of the eight spores illustrated by Spegazzini is provided with a longitudinal septum, this feature is not mentioned in either the generic or species description, and it cannot be regarded as a general characteristic.

FAMILY II. *Chaetothyriaceae* Th. emend. E. Fisher *non Chaetothyrieae* Theiss. and Syd. 1917.

The components of this family were determined after a critical survey of all the genera, which have been referred either to the Coccodineae v. Höhn. or to the *Chaetothyrieae* Th.

A small group with ascocarps of soft, membranous texture and ostiolate structure was segregated from the Capnodiaceae by von Höhnel (1909b). He (1915, 1918) made subsequent additions to this group, and it was eventually named the Coccodineae.

Unfortunately, the genus *Coccodinium* appears to be an extraneous member. It does not exhibit the superficial habit of growth which is so characteristic of 'sooty moulds'. The subiculum of *Coccodinium Bartschii* is developed in the host periderm, according to von Höhnel (1918); and the description of the same fungus given by Millardet (1870, Fig. 18) also indicates that the mycelium is embedded in the tissue of the host.

The name Coccodineae is invalidated by the exclusion of the genus *Coccodinium*. However, with the removal of this genus, the Coccodineae v. Höhn. becomes identical with the Chaetothyriaceae Th.; and the latter name will be adopted in this paper.

The Chaetothyriaceae was originally described by Theissen (1913, p. 496) as a section of the Hypocreaceae; but later, in the 'Synoptische Tafeln' of Theissen and Sydow (1917), it was included in the family Capnodiaceae v. Höhn. Theissen and Sydow enlarged the Chaetothyriaceae Th. very considerably, but many of the additional genera appear to be unrelated to the members of Theissen's original group, and they are excluded from the Chaetothyriaceae as defined in the present paper.

A. Spores muriform-septate.

- | | | |
|-------------------------------|-----------|---|
| Spores hyaline to light brown | | Phaeosaccardinula (P. Henn.)
char. emend. v. Höhn.
syn. Limacinula Sacc.
Treubiomycetes v. Höhn. |
|-------------------------------|-----------|---|

B. Spores phragmoseptate.

- | | |
|--|--|
| (a) Spores brown | Setella Syd. |
| (b) Spores hyaline to light olivaceous | Chaetothyrium (Speg.)
char. emend. Theiss.
syn. Zukalia Sacc.
Aithaloderma Syd. |
| (i) spores elliptical oblong | Actinocymbe v. Höhn. |
| (ii) spores thread-like | |

Discussion of Genera

An historical survey of the genera included in the Chaetothyriaceae Th. emend. indicates a general confusion in the conception of ascocarp-shape. All genera, excepting *Setella* Syd., have been described from species which were originally referred to the Microthyriaceae Sacc. They were subsequently removed from this family because it was found that their ascocarps did not conform to a characteristically flat, shield-like shape. Consideration will first

be given to the type-genus of the Chaetothyriaceae, and then to the other three genera in order of priority.

(i) *Chaetothyrium* Speg. *char. emend.* Theiss.

This genus was described by Spegazzini (1888) as a member of the Microthyriaceae, but re-examination of the type-species, *Ch. guaraniticum*, caused Theissen (1913) to question the accuracy of Spegazzini's classification.

Theissen considered the type-specimen of *Ch. guaraniticum* Speg. to be too small for sectioning, and for more detailed observation of ascocarp-structure he confined his attention to a very similar fungus, which he named *Chaetothyrium Rickianum*. With the aid of transverse sections Theissen observed that a hemispherical subiculum covered the ascocarp, which was thereby readily distinguished from the flat type of fructification so characteristic of the Microthyriaceae.

More recently, Bitancourt (1936) has re-examined and successfully sectioned the type-specimen of *Ch. guaraniticum* Speg. By the demonstration of a hemispherical ascocarp in the actual type-species of the genus, Bitancourt's investigation (Pl. I, Fig. D) gave confirmation to Theissen's conception of ascocarp-structure in *Chaetothyrium*.

The description given by Theissen may therefore be accepted as accurate, and his definition of the genus *Chaetothyrium* will be adopted here.

Synonymy. The genus Zukalia was created (Saccardo, 1891, p. 431) for the reception of a hyaline-spored fungus known as *Meliola* sp. Any affinity between Zukalia and Meliola was discredited, however, by von Höhnel's re-examination (1910b, p. 916) of the type-specimen, *Z. loganiensis* Sacc. et Berl. Although he was unable to find any ripe fructifications, von Höhnel identified *Z. loganiensis* as an undoubted member of the Naetrocymbeae (Coccodineae). Later Theissen and Sydow (1917) identified Zukalia Sacc. with the older genus *Chaetothyrium* Speg.; and the synonymy established by them will be accepted in this paper.

The nomenclature of *Chaetothyrium* is complicated by the fact that just six months prior to Theissen's emendation a closely related species was described by Sydow (1913, p. 257) as the type of a new genus, *Aithaloderma*. In the 'Synoptische Tafeln' of Theissen and Sydow (1917) both genera were retained. These authors attributed setulose mycelium and fructifications to *Chaetothyrium*, while *Aithaloderma* was distinguished by the restriction of bristles to the apical region of the fructifications. However, in *Chaetothyrium Citri* the writer has found the development of bristles to be a variable character, and, moreover, this is regarded by both von Höhnel (1918) and Petrak (1929) as an unreliable feature for generic distinction.

Fraser (1935c), recognizing the arrangement of bristles to be unreliable, suggested that the genus *Chaetothyrium* should be distinguished from *Aithaloderma* by the absence of pycnidia and by relatively large ascostromata. According to Fraser the ascostromata of *Chaetothyrium* are usually more than

200μ in diameter and the fructifications of Aithaloderma average 100μ . Not only does this appear to be an inadequate basis of separation, but it is invalidated by the fact that in the original description of the genus Chaetothyrium the perithecial dimensions are given as $100-50\mu$ in diameter. It is suggested, therefore, that the genus Aithaloderma is synonymous with Chaerothyrium.

Some reference to Chaetothyridina Th. is involved in a historical survey of the genus Chaetothyrium. The former genus was created by Theissen (1913) for the reception of *Chaetothyrium musarum* Speg., a species with bicellular ascospores. Theissen attributed hemispherical ascocarps to *Chaetothyridina musarum* (Speg.) Theiss.; however, when Bitancourt (1936, Plate II, Fig. 6) examined the type-specimen flat shield-like ascocarps were demonstrated. Consequently, the genus Chaetothyridina Th. cannot be included in the family Chaetothyriaceae Th. emend., and it appears to be more naturally placed in the Microthyriaceae Sacc.

(ii) *Phaeosaccardinula* P. Henn. char. emend. von Höhn.

The type-species *Ph. diospyricola* was described by Hennings (1905, p. 67) as a member of the Microthyriaceae which possessed perithecia of the characteristic 'scutellato-dimidiata' shape.

When von Höhnel (1910b) re-examined the type-specimen of Hennings's fungus he identified it as a typical *Limacinula*. He described perithecia enveloped in a stroma, the upper part of which was thicker than the rest.

Synonymy. Later, von Höhnel (1915) reverted to the generic name *Phaeosaccardinula* because it was described four months prior to Saccardo's establishment of the sub-genus *Limacinula*. The former genus was included in the Naetrocymbeae (Coccodineae) and *Limacinula* was listed as a synonym.

It is proposed to follow the suggestion of Petrak (1929) and to regard *Phaeosaccardinula* and *Treibiomycetes* v. Höhn. (1909a) as generically indistinguishable. The latter genus is only to be distinguished from *Phaeosaccardinula* by its setulose perithecia, and, as already indicated, the development of bristles cannot be regarded as a reliable basis for generic separation.

Petrak (1929) has extended the synonymy farther. He has included *Phaeosaccardinula* in the genus *Chaetothyrium*, because from his point of view spore-septation is too variable to be used as a feature of generic distinction. However, so far as my observations have extended, the type of spore-septation has proved to be a constant character, and it is proposed to retain the genus *Phaeosaccardinula* for the reception of species with muriform-septate spores.

(iii) *Actinocymbe* v. Höhn.

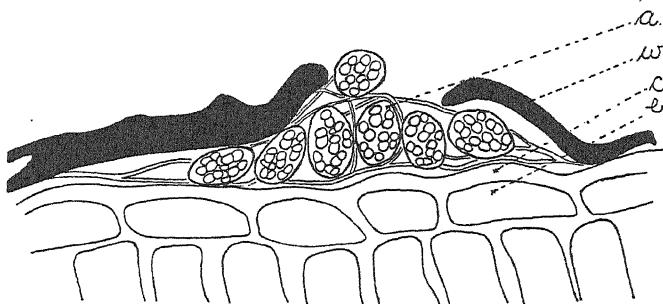
The genus *Actinocymbe* was also described from a species which was originally included in the Microthyriaceae. The type-species was described by Hennings (1908) under the name *Actiniopsis separato-setosae*.

When von Höhnel (1911) emended Hennings's description, he denied the

flat shield-like shape, hitherto attributed to the ascocarps of *Actiniopsis separato-setosae* P. Henn., and its removal from the Microthyriaceae, was thereby necessitated. The genus *Actinocymbe* was then created to accommodate this species.

(iv) *Setella* Syd.

The history of *Setella* Syd., unlike that of the other genera, does not indicate any connexion with the Microthyriaceae, as the type-species, *S. disseminata*,



TEXT-FIG. 1. Median longitudinal section through an ascocarp of *Dimerosporium Veronicae*.
w, wall of ascocarp; a, ascus; c, cuticularized wall, of leaf-epidermal cell, e. $\times 375$.

was described (Sydow, 1916, p. 359) after the Neatrocymbeae (Coccodineae) v. Höhn. and Chaetothyrieae Th. were firmly established.

FAMILY III. *Microthyriaceae* Sacc.

The Microthyriaceae Sacc. has not been studied in sufficient detail for a generic classification to be offered.

Some specimens have been examined, however, and a means of differentiating this family from the closely related Chaetothyriaceae has been considered. As exemplified by the species *Chaetothyrium Citri* (Arn.) nov. comb. (see Pl. XII, Fig. 3)¹ and *Dimerosporium Veronicae* (Lib.) Nob. Text-fig. 1, respectively, the ascocarps may be described as hemispherical in the Chaetothyriaceae, and as flattened in the Microthyriaceae. Furthermore, the Microthyriaceae may usually be identified by a characteristic radial dehiscence of the ascocarp wall. This feature is well illustrated by the specimen named by Fraser, *Aithaloderma viridis* (see Pl. XII, Fig. 7).

FAMILY IV. *Trichopeltaceae* Th.

A very satisfactory classification of the genera included in this family has been suggested by Theissen (1914, p. 630).

FAMILY V. *Perisporiaceae* Fr.

As the present study is confined to saprophytic 'sooty moulds' no attempt

¹ A microscopic preparation of the type-specimen, which was made available to the writer by the courtesy of Dr. Arnaud, forms the subject of this photomicrograph.

will be made to amplify the foregoing description of the Perisporiaceae (see above).

FAMILY VI. *Atichiaceae* Millardet emend. Raciborski.

The history of this family, which constitutes an aberrant group of fungi, has already been outlined by Cotton (1914).

It comprises only two genera, which may be separated as follows:

Propagula restricted to particular areas of the

thallus

ATICHIA Flotow.

emend. Rac.

syn. *Seuratia* Pat.

pro parte.

Propagula uniformly distributed over the surface

of the thallus

PHYCOPSIS Mang.

and Pat.

syn. *Seuratia* Pat.

pro parte.

It was as early as 1850 that Flotow described the type-genus *Atchia*. Later, Mangin and Patouillard (1912) created the genus *Phycopsis* for the inclusion of species characterized by a spherical thallus bearing the asexual reproductive organs or propagula uniformly distributed over its surface. Subsequent investigators, including Arnaud (1925), Sydow (1926), and Boedijn (1931), have suggested the two converse characters, a stellate or lobed thallus, and the localization of the propagula in particular areas, as diagnostic of the genus *Atchia*.

Observations made by the writer indicate that the distribution of the propagula is more reliable than the shape of the thallus as a generic character. On examination of the Atichiaceae represented in the herbarium at the Royal Botanic Gardens, Kew, it was found that the thallus of *A. glomerulosa* is not always deeply lobed. In fact, as noted by Millardet (1870), the young thalli are usually spherical in shape. Therefore in this paper the genera *Atchia* and *Phycopsis* are differentiated according to the distribution of the propagula only.

The genus *Seuratia*, which was described by Patouillard (1904), fell into synonymy when von Höhnel (1910) identified the type-species with *Atchia Millardetii* Rac. Nevertheless, for some years French mycologists, including Mangin and Patouillard (1912), continued to recognize *Seuratia* Pat., and they referred to this genus all species for which an ascigerous stage had been described.

Conclusion

It is evident that the term 'sooty mould' has been applied to fungi which from a taxonomic point of view constitute a very heterogeneous group of genera.

The fungi which are endemic to a 'sooty mould' habitat may be referred to six separate families of the Ascomycetes. The differentiation of these families

requires that the inadequate cytological data available should be supplemented by other diagnostic characters. A useful means of identification is provided by the macroscopic appearance of the covering which is produced on the surface of the host. Also, a knowledge of microscopic characters, including the septation of the hyphae, the presence or absence of hyphopodia, and the shape of the ascocarps, is valuable.

Within each family the separation of genera may usually be established by reference to ascospore characters such as septation and colour. An exception is provided by the Atichiaceae in which ascospore characters are remarkably uniform; the two genera may then be distinguished, according to the type of conidial development.

Furthermore, the 'sooty mould' fungi may be classified in two categories according to their mode of nutrition:

- (a) Epiphytes, which grow purely saprophytically on 'honey dew'-covered plants. All species are capable of growth on culture-media. 'Sooty mould' endemics, as well as unspecialized components, are included in this category.
- (b) Ectoparasites, which produce an abundant superficial mycelium but also derive nutriment from the host-plant. These species have not yet been grown on culture-media.

II. CULTURAL STUDY

Introduction.

As early as 1878 Zopf grew in culture a mixture of 'sooty mould' fungi which he described as the 'conidienfrüchte von *Fumago*.' 'Pure culture' methods were employed for the first time by Neger (1918). These methods have been adopted by subsequent investigators (Tengwall, 1924; Sawhney, 1927; van Beyma, 1931; Fisher, 1933; Fraser, 1933), and a considerable number of 'sooty mould' components have now been isolated and grown in culture. Fraser (1934) examined the nitrogenous and carbohydrate requirements of species isolated from the 'sooty moulds' of New South Wales. More recently (1937) the same author tested the resistance of these fungi to extremes of temperature and to desiccation, but the influence of these climatic factors on the rate of growth was not studied.

The writer has grown, under controlled conditions of temperature and atmospheric humidity, the following species:

'Sooty mould' endemics	<i>Phycopsis</i> sp. <i>Capnodium salicinum</i> Mont. <i>Fumagospora</i> sp. <i>Hendersoniella</i> sp. <i>Antennularia</i> sp. <i>Microxyphium</i> sp. <i>Microxyphium Leptospermi</i> E. Fisher <i>Pleospora herbarum</i> (Pers.) Rabh.	{	Atichiaceae Capnodiaceae
Unspecialized 'sooty mould' component			Phomaceae
			Sphaeriaceae

These species were isolated from epiphytic 'sooty moulds' occurring near Melbourne; for the most part they have been previously described (Fisher, 1933). The fungus referred to the genus *Phycopsis*, however, constitutes a new species and a detailed description will appear in another paper.

The mother-culture of *Capnodium salicinum* was grown from an ascospore; but five of the endemic species have been referred to form-genera, as no connexion with an ascigerous stage was established. The cultural characters of the latter species closely resemble those of *Capnodium salicinum*; furthermore, Fraser (1935b) has associated similar pycnidial forms with Capnodiaceae-genera. However, although these fungi were grown on a variety of media at different temperatures, only the pycnidial stage developed, and they have therefore been assigned to the family Phomaceae.

Effect of temperature on growth.

Curves of growth. By courtesy of the Director of the Low Temperature Research Station, Cambridge, facilities were made available for testing growth at various temperatures. The experiment was conducted in three similar sections, in each of which Petri-dish cultures of the eight species listed above were exposed in triplicate to 10°, 15°, 18°, 20°, and 25° C. respectively.

A culture-medium of 2 per cent. agar containing 5 per cent. honey was used. The inoculum consisted of hyphae taken from the margin of a Petri-dish culture approximately one month old. In the case of *Pleospora herbarum* a disc of mycelium was cut by means of a cork-borer 1·5 mm. in diameter, but a smaller inoculum measuring approximately 0·5 sq. mm. was found more satisfactory for the more slowly growing species.

Adopting the linear method of growth-measurement the average diameter of each colony was noted at stated intervals. Owing to the characteristically slow growth of the endemic species, measurements which were taken every four or five days extended over a period of approximately six weeks. However, at temperatures of 18–25° C., *Pleospora herbarum* will cover a Petri dish (7 cm. diameter) in ten days, and so it was necessary to measure this species more frequently.

These data which occupy twenty-four tables and over one hundred growth-curves could not be published in their entirety. They are, however, included in the copies of the Thesis which have been deposited in the University Libraries of Cambridge and Melbourne.

The measurements recorded for *Capnodium salicinum* in the second section of this experiment are quoted here as illustrative examples. The average diameter of each colony and the measurement for each group of cultures exposed to the same temperature have been tabulated (see Table I). These mean measurements are also represented by the ordinates of the growth-curves which are illustrated in Text-Fig. 2.

TABLE I
Temperature-growth Relations of Capnodium salicinum.
Series B. commenced 13 Feb. 1936

Tempera- ture.	No. of culture.	Average diameter of colony, measured in centimetres.									Average growth 4 days.
		18/2/36.	22/2/36.	27/2/36.	2/3/36.	6/3/36.	10/3/36.	14/3/36.	18/3/36.	23/3/36.	
10° C.	i	0·2	0·32	0·37	0·42	0·45	0·5	0·53	0·55	0·58	0·06 cm.
	ii	0·15	0·25	0·35	0·36	0·45	0·52	0·52	
	iii	0·17	0·32	0·38	0·43	0·52	0·58	0·67	0·75	0·77	
	Mean of i and iii	0·18	0·32	0·37	0·42	0·48	0·54	0·6	0·65	0·67	
	i	0·35	0·57	0·86	1·10	1·30	1·45	1·55	1·62	1·65	
	ii discarded	
15° C.	iii	0·32	0·55	0·85	1·08	1·30	1·47	1·57	1·70	1·72	0·23 cm.
	Mean of i and iii	0·33	0·56	0·85	1·09	1·30	1·46	1·56	1·66	1·68	
	i	0·38	0·72	1·17	1·50	1·82	2·05	2·35	2·47	2·65	
	ii	0·37	0·7	1·15	1·45	1·8	2·05	2·30	2·45	2·55	
	iii	0·42	0·72	1·17	1·52	1·82	2·10	2·32	2·47	2·65	
	Mean	0·39	0·71	1·16	1·49	1·81	2·06	2·32	2·40	2·62	
18° C.	i	0·4	0·77	1·18	1·52	1·82	2·03	2·17	2·27	2·37	0·34 cm.
	ii	0·47	0·77	1·25	1·55	1·87	2·1	2·22	2·42	2·52	
	iii	0·42	0·80	1·17	1·45	1·7	1·92	1·97	
	Mean of i and ii	0·43	0·77	1·21	1·53	1·84	2·06	2·19	2·34	2·44	
	i	0·15	0·3	0·62	0·74	0·85	1·0	1·05	1·1	1·1	
	ii	0·25	0·42	0·67	0·82	1·0	1·17	1·2	1·22	1·22	
20° C.	iii	0·25	0·4	0·57	0·67	0·77	0·9	1·12	1·3	1·4	0·34 cm.
	Mean	0·22	0·37	0·62	0·74	0·87	1·02	1·12	1·21	1·24	
	
25° C.	iii	0·13 cm.
	Mean	
	

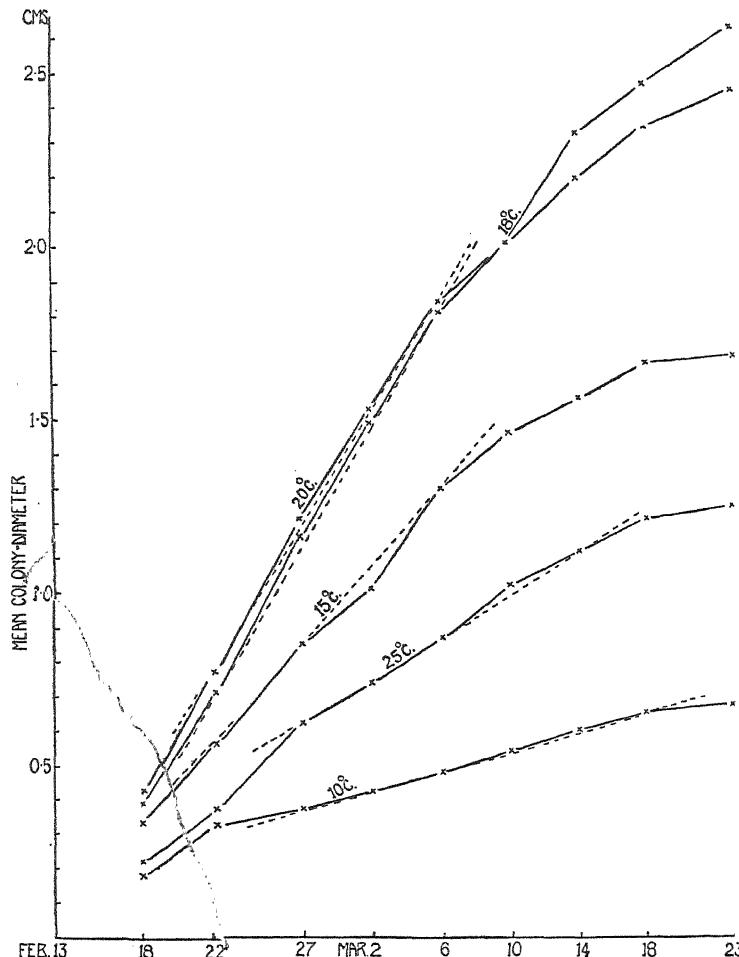
Following the method suggested by Tomkins (1929, p. 381), a straight line was drawn through each curve in the region of regular growth; and from the slope of these lines the average growth-rates have been derived. In this way, growth has been measured for each of the eight species under consideration (see Table II). However, owing to the diversity in the inherent growth-rates of these species, the method suggested by Fawcett (1921, p. 211) has been adopted, and relative values in which the maximum growth-rate is represented by unity are recorded (see Table II and Text-fig. 3).

According to the form of these growth-temperature curves the species examined may be arranged in four groups. For *Phycopsis* sp. and *Fumago-spora* sp., the growth-rate rises gradually with each increase in temperature until the optimum is reached; above this a rapid decline in the rate of growth occurs. The growth of *Phycopsis* sp. is entirely inhibited at 25° C.; and, from the slope of the curve in the supra-optimal region, it may be inferred that 21° C. would approximate to the maximum temperature for growth.

The growth of *Hendersoniella* sp., *Microxyphium* sp., and *Capnodium salicinum*, also falls off rapidly when the optimum temperature is exceeded, but these species are almost as sensitive to sub-optimal as to supra-optimal temperatures.

Microxyphium Leptospermi and *Antennularia* sp. may be distinguished by the fact that there is no sudden decrease in the rate of growth at supra-optimal temperatures. It is therefore likely that conditions of high temperature will be more favourable to the growth of these fungi than to those previously mentioned.

Pleospora herbarum is unique amongst the species examined, in that each increase of temperature within the range tested caused an acceleration in the rate of growth.



TEXT-FIG. 2. Curves of growth for *Capnodium salicinum* at 10°, 15°, 18°, 20°, and 25° C.

Resistance to low temperatures.

Petri-dish cultures of the eight species were sown and were immediately exposed in triplicate to a temperature of -10° C. Approximately eighteen hours later, freezing of the medium was precipitated by touching it with a sterilized needle; the dishes were allowed to remain at this temperature for a period of three days, after which they were transferred to 18° C.

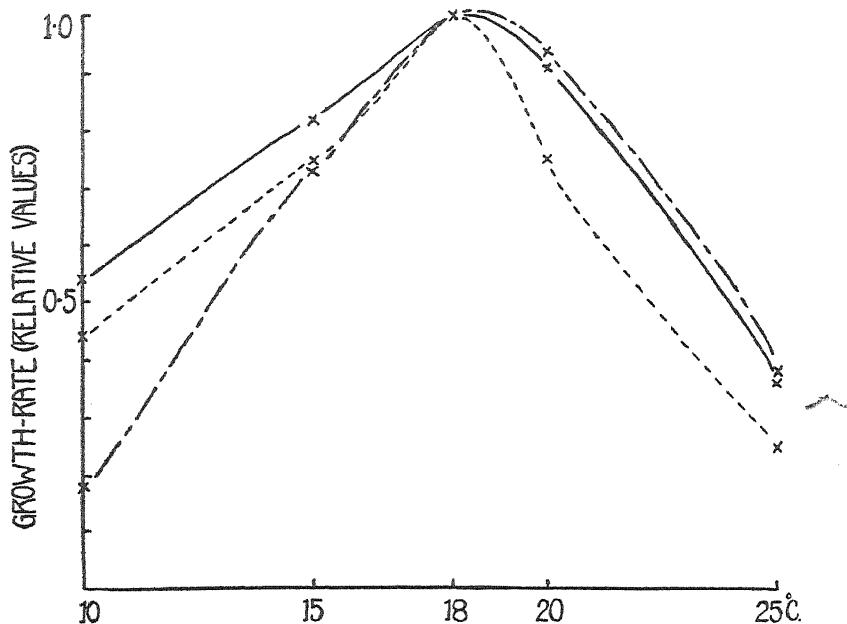
Pleospora herbarum did not show any sign of growth after ten days' exposure

TABLE II
Average Growth Four Days Measured in Centimetres

Fungus.	Series	No.	10° C.	15° C.	18° C.	20° C.	25° C.	Optimum temperature.
<i>Phycopsis</i> sp.	A.		0.11	0.18	0.15	0.05	none	15° C.
	B.		0.16	0.17	0.10	0.08	„	
	C.		0.16	0.20	0.16	0.08	„	
	Mean		0.14	0.18	0.14	0.07	„	
	Relative value		0.78	1.0	0.78	0.39	—	
<i>Capnodium salicinum</i>	A.		0.04	0.24	0.33	0.29	0.19	18.5° C.
	B.		0.06	0.23	0.34	0.34	0.13	
	C.		0.07	0.27	0.35	0.34	0.08	
	Mean		0.06	0.25	0.34	0.32	0.13	
	Relative value		0.18	0.73	1.0	0.94	0.38	
<i>Fumagospora</i> sp.	A.		0.17	0.38	0.35	0.34	0.03	20° C.
	B.		0.13	0.30	0.36	0.37	0.08	
	C.		0.19	0.33	0.38	0.42	0.05	
	Mean		0.16	0.34	0.36	0.38	0.05	
	Relative value		0.42	0.89	0.95	1.0	0.13	
<i>Hendersoniella</i> sp.	A.		0.06	0.11	0.15	0.09	0.04	18° C.
	B.		0.06	0.11	0.15	0.12	0.03	
	C.		0.08	0.14	0.18	0.14	0.04	
	Mean		0.07	0.12	0.16	0.12	0.04	
	Relative value		0.44	0.75	1.0	0.75	0.25	
<i>Antennularia</i> sp.	A.		0.09	0.18	0.24	0.26	0.17	20° C.
	B.		0.08	0.18	0.26	0.28	0.22	
	C.		0.09	0.19	0.22	0.26	0.22	
	Mean		0.09	0.18	0.24	0.27	0.21	
	Relative value		0.33	0.67	0.89	1.0	0.78	
<i>Microxyphium Leptospermi</i>	A.		0.13	0.19	0.22	0.22	0.20	19° C.
	B.		0.11	0.19	0.22	0.22	0.20	
	C.		0.12	0.17	0.22	0.22	0.17	
	Mean		0.12	0.18	0.22	0.22	0.19	
	Relative value		0.54	0.82	1.0	1.0	0.86	
<i>Microxyphium</i> sp.	A.		0.06	0.08	0.10	0.10	0.04	18° C.
	B.		0.06	0.09	0.10	0.11	0.03	
	C.		0.06	0.10	0.12	0.09	0.06	
	Mean		0.06	0.09	0.11	0.10	0.04	
	Relative value		0.54	0.82	1.0	0.91	0.36	
<i>Pleospora</i> herbarum	A.		0.25	0.47	0.60	0.70	0.72	25° C. or higher
	B.		0.27	0.47	0.62	0.65	0.70	
	C.		0.32	0.47	0.62	0.65	0.67	
	Mean		0.28	0.47	0.61	0.67	0.70	
	Relative value		0.4	0.67	0.87	0.96	1.0	

¹ The daily growth-rate is given for this species.

to 18° C., but all the other species grew normally. The latter were then replaced at -10° C. and the medium was frozen as before. Although the hyphae were now in intimate contact with the frozen medium they were not



TEXT-FIG. 3. Growth-temperature curves for *Microxyphium* sp. —, *Hendersoniella* sp. - - -, and *Capnodium salicinum* - - -.

killed by exposure to this temperature for a period of three days, as normal growth occurred when the cultures were again restored to 18° C.

Discussion.

The abundance of 'sooty mould' endemics throughout the warmer climatic zones has led to the assumption that high temperatures are favourable to the growth of these fungi. However, the comparatively low temperature-optima of the species considered in this paper do not tend to confirm that view. *Phycopsis* sp. grows best at 15° C. and for the other species the optima lie between 18° C. and 20° C. Moreover, the growth of these fungi, with the exception of *Microxyphium Leptospermi* and *Antennularia* sp., declines rapidly with supra-optimal temperatures.

A seasonal development of 'sooty moulds' appears to be a general phenomenon. Fraser (1933, p. 384) recorded that the Australian 'sooty moulds' of N.S.W., although common throughout the year, are more abundant during the winter months; and in the south of France, Arnaud (1910b, p. 244) observed that summer conditions are not favourable to 'fumagine' development.

Fraser (1933, p. 390) concluded that the summer decrease of 'sooty moulds'

was due to the disappearance of fungi such as *Alternaria* spp. and *Epicoccum* spp., which she classified as ephemeral components.

The temperature requirements of the fungi examined by the writer suggest, however, that a different interpretation should be given to the role played by the various components in the establishment of seasonal 'sooty moulds'. The growth of endemic species on culture-media was adversely affected by high temperatures. On the contrary, the growth of an unspecialized component, *Pleospora herbarum*, was neither retarded by a temperature of 25° C., nor, when compared with the other species examined, was it resistant to cold. Generalizations concerning the temperature requirements of the unspecialized 'sooty mould' components cannot be made when only one species has been tested. Nevertheless, it is a noteworthy fact that, in regions where deciduous trees constitute a large part of the vegetation and where the 'sooty moulds' of necessity exhibit summer growth, such cosmopolitan fungi almost invariably form the sole constituents.

It is likely therefore that the greater abundance of 'sooty moulds' during the winter, which has been observed in warm temperate regions, is due to the renewed growth of the 'sooty mould' endemics themselves, and not the unspecialized species. The establishment of this hypothesis requires confirmation by field observation of 'sooty mould' development. The composition of artificially resynthesized 'sooty moulds' could be controlled and the seasonal development of the various types of mould compared.

Effect of humidity on growth.

Curves of growth. The necessity for maintaining a constant temperature if humidity is to be regulated in a reliable way has already been indicated by Tomkins (1929, p. 393). After reviewing the temperature-optima of the species to be considered, 18° C. was chosen as the temperature most convenient for examining their humidity requirements.

A satisfactory method for growing fungi under conditions of controlled atmospheric humidity was described by Tomkins (1929), and it has been adopted by the writer. Cultures which were sown on thin films of honey-agar were suspended within closed glass jars in which the humidity of the air was controlled by means of NaCl solutions. Each species was exposed to a variety of relative humidity (R.H.) ranging from 100–90 per cent.

It has been impossible to publish the daily measurements of colony-diameter, but they are recorded in the copies of my thesis. However, the rates of growth which were determined from these measurements are cited here (see Table III). The relative values, in which the maximum rate of growth is expressed as unity, are also represented by the ordinates of the growth curves, as exemplified in Text-fig. 4.

DISCUSSION

Notwithstanding the exposed nature of their habitat, the growth of the

TABLE III

Effect of Atmospheric Humidity on Growth at 18° C.

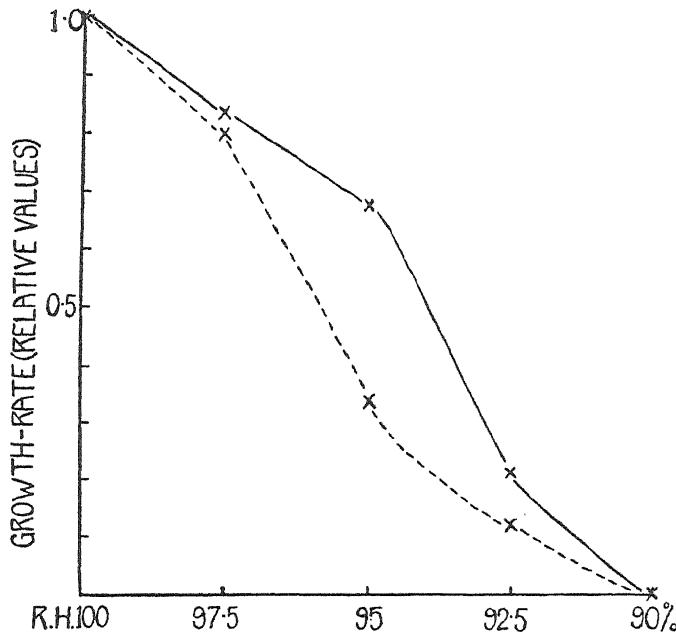
Fungus	Growth.	R.H. 100 per cent.	R.H. 99 per cent.	R.H. 98 per cent.	R.H. 97·5 per cent.	R.H. 96 per cent.	R.H. 95 per cent.	R.H. 92·5 per cent.	R.H. 90 per cent.
<i>Phycopsis</i> sp.	Aver. rate 2 days	0·15 mm.	0·17 mm.	0·05 mm.	..	0·0 mm.	none
	Relative value	0·88	1·0	0·29	..	0·06	0
<i>Capnodium</i> <i>salicinum</i>	Aver. rate 2 days	1·05 mm.	0·87 mm.	..	0·72 mm.	0·22 mm.	none
	Relative value	1·0	0·83	..	0·68	0·21	0
<i>Fumagospora</i> sp.	Aver. rate 2 days	1·17 mm.	0·97 mm.	..	0·55 mm.	0·22 mm.	none
	Relative value	1·0	0·83	..	0·47	0·19	0
<i>Hendersoniella</i> sp.	Aver. rate 2 days	0·65 mm.	0·52 mm.	..	0·22 mm.	0·08 mm.	none
	Relative value	1·0	0·8	..	0·34	0·12	0
<i>Antennularia</i> sp.	Aver. rate 2 days	0·95 mm.	..	0·85 mm.	..	0·70 mm.	none
	Relative value	1·0	..	0·89	..	0·74	0
<i>Microxyphium</i> <i>Leptospermi</i>	Aver. rate 2 days	0·72 mm.	0·37 mm.	..	0·1 mm.	none	..
	Relative value	1·0	0·53	..	0·14	0	..
<i>Microxyphium</i> sp.	Aver. rate 2 days	0·62 mm.	0·4 mm.	..	0·27 mm.	0·1 mm.	none
	Relative value	1·0	0·64	..	0·43	0·16	0
<i>Pleospora</i> <i>herbarum</i>	Aver. rate 2 days	4·1 mm.	2·05 mm.	..	1·4 mm.	none	..
	Relative value	1·0	0·5	..	0·34	0	..

'sooty mould' fungi appears to be very sensitive to variations in the degree of atmospheric humidity. In fact, the humidity requirements of the species considered here are very similar to those of other fungi; their growth limits are narrow. The lower limit under the conditions of the experiment being 92·5 per cent. relative humidity at 18° C. Furthermore, it has not been possible on the basis of humidity growth-rate relations to distinguish *Pleospora herbarum*, which is an unspecialized 'sooty mould' component, from the endemic species.

CONCLUSION

When the geographic distribution of 'sooty moulds' is considered in the light of the data presented in this paper, it is apparent that factors other than temperature and atmospheric humidity are also involved.

The absence of species belonging to the Capnodiaceae is a conspicuous feature of the 'sooty moulds' occurring in cool temperate climates (Neger, 1918; Tengwall, 1924); and yet the growth of the species examined by the writer does not appear to be favoured by high temperatures. However, the development of a deciduous forest vegetation is characteristic of the cool temperate regions; also, the Conifers, though evergreen, are characterized by



TEXT-FIG. 4. Growth-humidity curves for *Capnodium salicinum* — and *Hendersoniella* sp. - - -.

a much reduced leaf-surface. Consequently substrata suitable for the growth of 'sooty moulds' are very limited during the winter. As this season is most favourable to the growth of the Capnodiaceae, it is not surprising that these species rarely occur in the cool temperate regions. On the other hand, it has been found that the growth of at least one unspecialized 'sooty mould' component is favoured by high temperatures. This evidence, correlated with the fact that the unspecialized species grow more rapidly than the endemics, suggests that the former are able to establish themselves on the new leaves, and so produce the sooty films which become prevalent towards the end of the summer.

In warm temperate regions, such as the countries bordering the Mediterranean and in Australia, an evergreen type of vegetation is predominant; and the slowly growing Capnodiaceae have an opportunity of establishing themselves during the winter. These fungi persist from season to season on the

perennial foliage, but the greatest development of mycelium occurs during the winter, when the sooty coverings become particularly conspicuous.

SUMMARY

1. After a critical analysis of previous investigations, the taxonomy of the 'sooty mould' fungi is discussed.
2. A classification is proposed by which these fungi are grouped according to the macroscopic appearance of their growth.
3. Diagnostic features of six families are given.
4. The Capnodiaceae v. Höhn. and Chaetothyriaceae Th. are emended, and keys are given for the identification of the genera included in these families.
5. Eight fungi isolated from epiphytic 'sooty moulds' have been grown under controlled conditions of temperature and atmospheric humidity.
6. The geographical distribution of epiphytic 'sooty moulds' is discussed in relation to the temperature and humidity requirements of the species examined. It is suggested that the scarcity of Capnodiaceae species in cool temperate climates may be correlated with the characteristic deciduous vegetation.

This work was carried out in the Botanical Departments of Cambridge and Melbourne Universities, and I wish to thank Professor F. T. Brooks and Associate-Professor E. I. McLennan for their interest in the work. I am also grateful to Miss E. M. Wakefield for advice in matters of taxonomy, and to Dr. R. G. Tomkins for help in connexion with the cultural study. For assistance in taking the photographs included in this paper I am indebted to Miss G. M. Fawcett.

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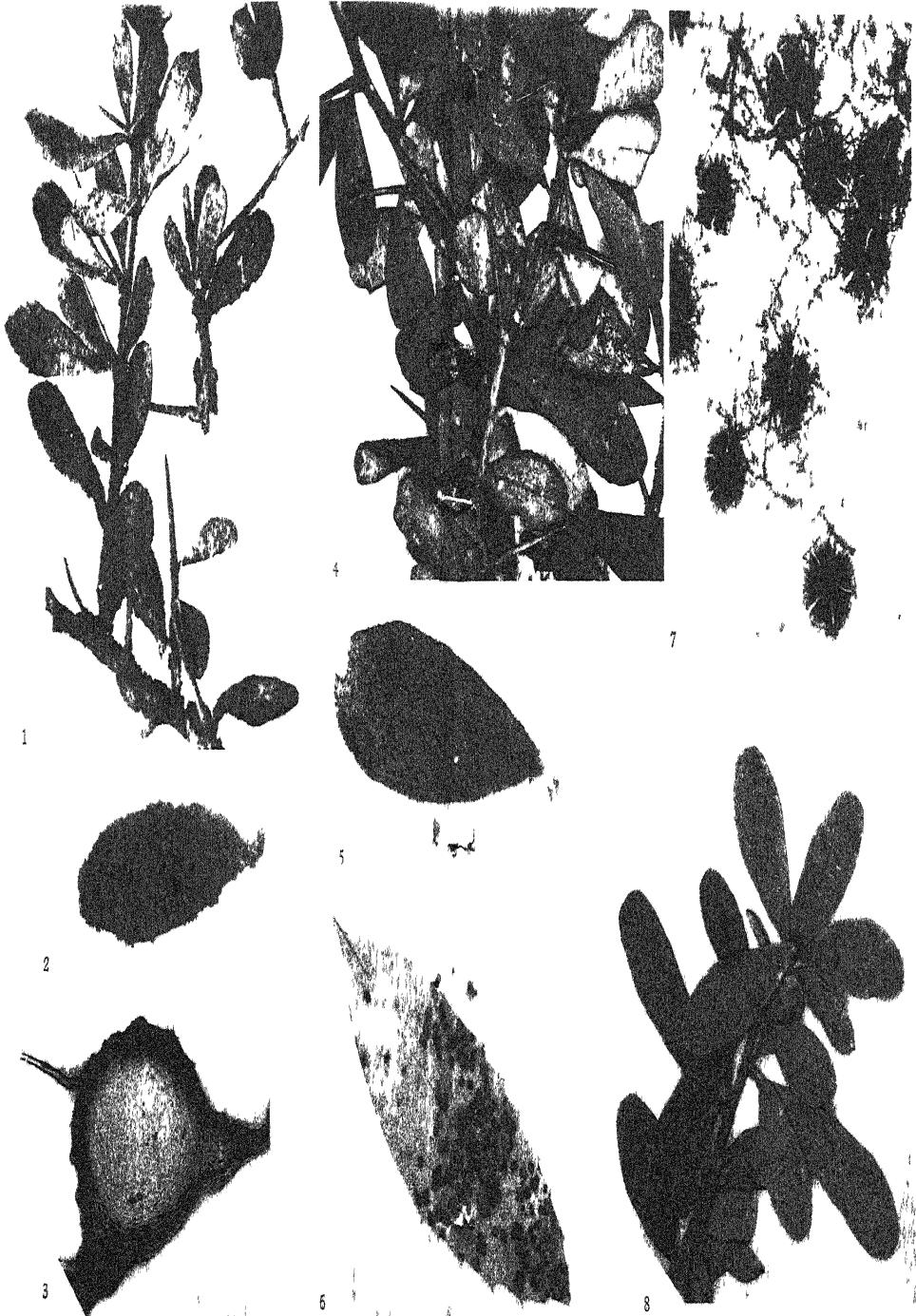
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EXPLANATION OF PLATE XII

Illustrating Dr. Eileen E. Fisher's paper on 'A Study of Australian "Sooty Moulds".'

- Fig. 1. A 'sooty mould' in which Capnodiaceae species are predominant, occurring on *Bursaria spinosa* Cav. $\times 3$.
- Fig. 2. One leaf of the specimen illustrated in Fig. 1. $\times 6$.
- Fig. 3. Median longitudinal section through an ascocarp of *Chaetothyrium Citri*. $\times 625$.
- Fig. 4. A 'sooty mould' in which a species of the Chaetothyriaceae (*Chaetothyrium Citri*) is predominant, occurring on *Bursaria spinosa*. $\times 3$.
- Fig. 5. One leaf of the specimen illustrated in Fig. 4. $\times 6$.
- Fig. 6. A species of the Perisporiaceae (*Meliola* sp.) on *Hedycarya angustifolia* A. Cunn. Natural size.
- Fig. 7. *Aithaloderma viridis* L. Fr. $\times 625$.
- Fig. 8. A species of the Atichiaceae (*Phycopsis* sp.) on *Bursaria spinosa*. $\times 3$.



The Absorption and Accumulation of Salts by Living Plant Cells

IX. The Absorption of Rubidium bromide by Potato Discs

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With eight Figures in the Text

SINCE Hoagland, Davis, and Hibbard (1926, 1928) employed bromides in their well-known experiments with Nitella these salts have been used in many investigations on salt absorption, and the principal variables which determine the absorption of bromide by various plant cells and tissues are now known.¹ The special qualities of the bromide ion give high precision to the results and enable some experiments to be carried out which are otherwise not feasible.

In the experiments with bromide it has usually been supplied as the potassium salt; data on *cation* absorption have been obtained only when the changes in the tissue or external solution were relatively large. A cation with qualities similar to those of the bromide ion would, however, facilitate many types of experimentation. Such a cation should be one which can be estimated quantitatively, and is not commonly present in cells, although readily absorbed when supplied. Like bromide the cation selected should not be metabolized or form insoluble compounds, but should accumulate in the cell-sap. These considerations restrict the possible choice to the alkali metals other than sodium or potassium.

Lithium was used by Birch-Hirschfeld (1919) and Kok (1932) in conjunction with a qualitative spectroscopic technique to indicate the movement of salt in plants. More recently Collander (1935), using Lundegårdh's quantitative spectroscopic technique, has examined the absorption of this element by the large cells of the Characeae. It is clear then that *rubidium* might be used to indicate translocation if a trustworthy method of estimation was available and the conditions for its absorption known.

In this paper a spectrographic method of estimating rubidium in plant

¹ Detailed references may be obtained from Steward (1935, 1937) or Hoagland and Broyer (1936).

extracts is described. Data are also provided which allow of a direct comparison between the factors which determine the absorption and accumulation of bromide and rubidium. This information provides the basis upon which the further use of rubidium in the investigation of either absorption or translocation in plants can be developed.

The amount of rubidium commonly present in soils and plants is so small that it can be neglected. From the effect of rubidium upon the absorption of anions, as observed by Hoagland, Davis, and Hibbard (1928), it may be presumed that the element, like potassium, is readily absorbed, and this is supported by actual determinations. Collander (1937) cites a case in which *Helianthus* seedlings were exposed to solutions of the same rubidium and sodium content and absorbed forty times as much of the former as of the latter. Collander also found that in seventeen species investigated the absorption of rubidium was closely correlated with that of potassium which it tended to exceed. The choice of rubidium for absorption studies is also satisfactory in that it is free from direct effects upon cells. Brenchley (1934), who cited the earlier work, concluded that rubidium sulphate in cultures of growing plants was neither beneficial nor harmful. Despite the reference to injurious effects of rubidium salts on growing plants, by Alten and Gottwick (1933) or to stimulating effects on micro-organisms by Lasnitzki and Szörenyi (1934), rubidium salts are not physiologically active. In the work to be described no adverse effects of rubidium salts upon the cells were observed. The lower yields observed by Scharrer and Schropp (1933) in cultures in which rubidium replaced potassium find a ready explanation in the need for potassium and do not suggest that rubidium salts are harmful. Heller, Peh, and Gürler (1934) noted the ready absorption of rubidium (detected spectrographically) by growing potato plants, as well as the relative increase in the degree of absorption from dilute solutions. The investigation of Brooks (1932 and 1935) on *Valonia* represents an attempt, based on a quantitative determination of rubidium, to utilize this element in the study of salt accumulation in plants.

The determination of rubidium in plant extracts.

The method adopted for the quantitative determination of rubidium in the presence of the other ions common in plant extracts was a spectrographic one. This method, however, was only employed when the available chemical methods had been found unsatisfactory.

The stannic chloride method, which was described by Strecker and Diaz (1925) and used by Fresenius (1931) for the determination of rubidium and caesium in natural waters, is the usual means of estimation. A simplified form of the stannic chloride method was used by Brooks in experiments on the uptake of rubidium by *Valonia*, and this author claims for it a degree of accuracy far greater than that described by the earlier workers. Detailed criticism of this method will be more appropriate elsewhere. The claim made

by Brooks (1932, p. 226) that in plant saps, which contain potassium in excess and are not free from other cations, rubidium can be determined so accurately that the mean of triplicate determinations 'is probably accurate to 1%' is at variance with our experience and with the evidence in the paper of Burkser, Milgewskaia, and Feldman (1930) to which Fresenius referred. The further problem presented by the removal of organic matter, a necessary prelude to the stannic chloride precipitation, need not be considered since this method of estimation was abandoned.

Spectrographic method.

Even if perfected, the estimation of rubidium in plant tissues by the stannic chloride method must be laborious. A spectrographic method was therefore employed, though the difficulties inherent in quantitative analysis by this means are numerous. Lundegårdh (1929, 1936) is notable among those who have applied the spectrograph to the investigation of mineral nutrition in plants. Only those factors which are essential to the successful quantitative determination of rubidium will receive attention here.

Rubidium has frequently been determined, at least semiquantitatively, in minerals and natural waters. In the investigation of Heller *et alia* (1934) the spectrograph was employed for the estimation of the approximate rubidium content of potato plants. A concentrated extract of the ash of the plants was fractionated by precipitation with sodium cobaltinitrite. The solution containing rubidium was fed directly into a flame of the bunsen type from a small porcelain boat by an ingenious method which used platinum wire as a 'wick' along which the solution moved by capillary action. Comparison with prepared standards was made using the lines 4215·6 and 4201·8, but, with the precautions described, the error was assessed at ± 20 per cent.

Quantitative use of the spectrograph depends upon the comparison of the intensity of lines produced by an unknown amount of the substance with those produced by a standard quantity. There are two main problems: first, to produce consistently lines of equal intensity from constant quantities of the substance; second, to compare accurately the intensities of lines produced by an unknown with those of a known amount. The first problem is the most difficult; the principal difficulty lies in the combustion of the sample. By rigid control of the causal factors the casual variations can be eliminated. This method of approach was preferred to the use of internal standards to compensate for the vagaries of individual combustions. The method of internal standards depends on the fact that both standard and unknown are affected in the same way, so that the final comparison can be based on the ratio between the unknown and the internal standard—a method to which Lewis (1933) adheres. The procedure to be described does not necessitate an internal standard and was elaborated from that briefly outlined by Ramage¹ (1929).

¹ Ramage apparently did not use his method except for the detection of metals in biological samples, with an approximate estimate of the order of their concentration, although

Although the arc is in more general use its advantages are not very apparent where plant samples are concerned, and they are offset by the greater control of the combustion which a gas flame permits. The burner was of the type used by Ramage and was made of two silica tubes, the outer one, carrying the coal gas, was 1·8 cm. in diameter and the inner one, carrying the oxygen, was made from capillary tube. The former projected 0·6 cm. above the latter, the

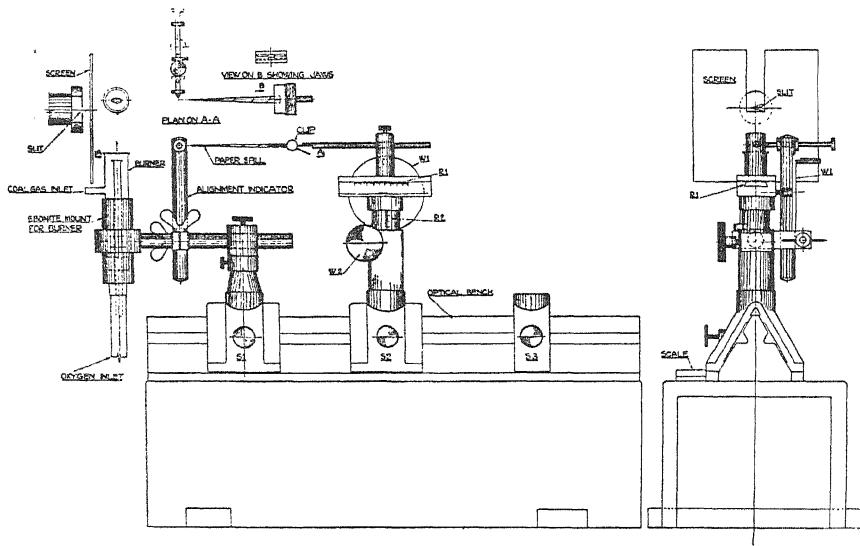


FIG. 1. Apparatus¹ for quantitative work on flame spectra of alkali metals.

orifice of which was flared so that the oxygen emerged from a rectangular aperture, 0·7 cm. by 0·2 cm. The actual combustion occurred behind a screen (see Fig. 1) which protected the spectrograph from direct light. The combustion conditions, to be described in detail later, resulted in the almost complete absence of a continuous spectrum; special importance is attached to this since the presence of a variable continuous spectrum complicates comparisons. The flame itself was standardized. The fine adjustment of the flow of coal gas was obtained by a screw clip or needle valve and the oxygen pressure was fixed by a reducing valve set to deliver at 30 lb. By these adjustments the flame conformed to arbitrary standard dimensions (e.g. over-all height and size of inner cone) which were kept constant throughout each series of com-

he believed it to be capable of considerable accuracy. Ramage described his procedure in the briefest manner and the method presented here differs from it in many ways, and also introduces the accurate estimation of the lines by photometry. The departures from the Ramage technique proved to be essential for accurate quantitative work and hence are described in some detail.

¹ Apparatus made to specifications by Precision Tool & Instrument Co. London.

bustions. The steady flame which is essential can best be obtained in a draughtless cellar. Even so the flame must be screened (by coloured glass) from the operator's breath, and rapid movements near the flame should be avoided.

Combustion technique.

In the method of Lundegårdh (1929, 1936) aqueous solutions are either introduced into an air-acetylene flame or spectra are produced from solutions by an 'immersion spark apparatus'. In the former method a complicated spraying device, which operates with amounts of 1·0 c.c. or 5·0 c.c., is used and the apparatus contains parts which must be cleaned between operations. The combustion technique here described was used with very small amounts of solution and was so rapid that all the operations for some twenty combustions could be completed in ten to fifteen minutes. The speed of the operations and the absence of all vessels which need cleaning is a noteworthy feature.

Only water extracts have been analysed. The material entered the flame dried on filter-paper after the method of Ramage (1929). Munktell's Swedish filtering-paper No. 00 was used and cut into rectangles 3 in. by 0·7 in. One edge was rolled preparatory to forming a spiral and a known amount of solution from a micropipette, calibrated to deliver 0·1, 0·075, 0·05 and 0·025 c.c., was run evenly over the middle of the paper. When using amounts greater than 0·05 c.c. this operation was performed in stages with periodic drying over a small flame or hot plate. The dry papers were then rolled spirally into a tight spill, at each extremity of which was a region free from the dried sample. To prevent the spiral from unwinding one end was flattened and twisted through 90°. Only those papers which were uniform in length and tightness were used and before combustion the papers were thoroughly dried in a vacuum oven.

Especial care was taken to ensure that all papers were burned in the same manner and in the same part of the flame. This was finally accomplished by the use of an apparatus designed for the purpose. This consists of three essential units, namely, an asbestos screen, the burner with its ebonite mount and heavy stand, and the unit which carries the paper spill.

The screen is placed between the flame and the spectrograph (Fig. 1, vertical section and end elevation) so that only the light which passes through a wide slit reaches the spectrograph. The best position for the screen is easily found by trial. It should be high enough to screen out the direct light from the slight luminous flame at the seat of the combustion and yet low enough to permit sufficient sensitivity to be obtained. The arrangement shown in Fig. 1 was satisfactory and the spectrograms obtained were almost completely free from continuous spectrum.

Both the silica burner and the paper spill are carried on heavy, rigid mounts attached to saddles (s_1 , s_2) which can slide along a rigid, triangular, optical

bench-bar to which they can be locked at will. The optical bench is precisely aligned with the spectrograph and the technique depends upon the strict alignment of the paper spill, the flame, and the slit throughout the combustion.

The paper spill is held by a clip with jaws made to hold the flattened end (see plan on A-A and view on B, Fig. 1). The clip is rigidly fixed to a shaft, which in turn is clamped to a centre post mounted on a small platform. For any given position of s_2 the shaft and paper spill can be moved smoothly and horizontally along the axis of the bench for a distance somewhat greater than the length of the spill. To attach the spill the saddle s_2 is withdrawn to a convenient position marked by a stop similar to s_3 . The paper is correctly aligned by means of the indicator (Fig. 1, see vertical section, end elevation and plan on A-A) the tip of which, in its forward position, denotes a point near to the flame, in alignment with the shaft and in the vertical plane of the centre tube of the burner and the slit of the spectrograph. The alignment indicator is withdrawn before the combustion begins. The passage of the paper into the flame is controlled by the wheel w_1 which operates the rack and pinion R_1 . The height at which combustion occurs can be varied at will by the coarse rack and pinion w_2 , R_2 , and a vertical adjustment of the burner can also be made when so desired.

The flame has an outer and colder zone within which is a sharply defined, hot, inner region at the edge of the oxygen cone. The saddle s_2 is moved along the bar rapidly from its first position until the tip of the spill reaches the hot zone of the flame and then, by the wheel w_1 , the approach of the spill can be so regulated that its brightly glowing tip, which is kept under observation through coloured glass, remains at the edge of the oxygen cone. In other words, the paper burns as quickly as it enters the flame, which should be neither so fast that it projects into the interior of the oxygen cone nor so slow that combustion occurs in the outer and colder zone.

The spectrograph.

The first instrument used was a Hilger Wavelength Spectrometer with camera attachment. With this instrument the rubidium lines 4215·6 and 4201·8 were separated on the plates by 0·8 mm. Later a Hilger medium quartz spectrograph fitted with an alternative glass train has been used and this gives a degree of dispersion approximately 1·5 times as great. The use of lenses to concentrate the maximum amount of light upon the slit was abandoned when it was realized that it could be a potent source of casual fluctuations in intensity; in the work to be described, accuracy was preferable to extreme sensitivity. As already described the flame was placed as near the slit as possible. That portion of the flame which became luminous was kept below the level of the slit (slit to top of burner 2·5 cm.) and further shielded by the screen described (Fig. 1). The width of the slit was 0·03 mm. The loss of sensitivity which this procedure entailed was more than counterbalanced by the accuracy gained and the almost entire absence of continuous spectrum. If all the precautions

mentioned here were observed, lines from quadruplicate samples on the same plate were not visibly different, even to the trained eye, whereas 10 per cent. differences in rubidium content produced differences which could easily be detected by eye.

The photographic plates.

Plates suitable for the different lines were selected. If an Ilford process plate was used the intensity of the lines 4215·6 and 4201·8 changed but slowly when exposed under the conditions described to 0·1 c.c. of solution of concentration below 0·001M and above 0·0032M. Between these two concentration limits the change of intensity was rapid. This sensitive range is the only one suitable for quantitative work. Infra-red plates which were very sensitive to the 7947·6 and 7800·3 lines are relatively insensitive to the 4215·6 and 4201·8 lines, and are only recommended for samples with very low rubidium content (less than 0·00025M). To make possible the simultaneous use of all of these lines, half the plate-holder of the wave-length spectrometer was charged with one plate (infra-red) and the other half with another strip of plate of suitable speed and sensitivity. The plates were developed singly by standard methods used to obtain the greatest contrast.¹

The procedure.

Each plate was exposed successively to 0·1 c.c. of the following standard solutions 0·0008M, 0·0016M, 0·0032M, 0·0064M, and these followed by spectra from 0·1 c.c. of eight unknown samples. The unknowns were then so diluted that they fell within the most sensitive range of the plates and spectrograms were again obtained from 0·05 or 0·1 c.c. of the diluted solutions on a plate which was calibrated by 8 to 10 spectrograms of standard amounts of rubidium.

The application of the microphotometer.

Examination of the plates by eye sufficed for all the experimental stages of the technique. It was, in fact, capable, though laborious, of yielding results of the requisite accuracy.² Greater speed and accuracy, and also confidence that personal factors did not affect the results, were secured by the use of the microphotometer; the instrument used was designed by Messrs. Adam Hilger, Ltd. The galvanometer scale deflexion given by the microphotometer was recorded for the background at fixed distances from each line measured. This deflection was invariably large and almost constant for a given plate, due to the almost entire absence of a continuous spectrum,³ and the precautions

¹ The developer used was hydroquinone, the final composition of which was 1 gr. hydroquinone, 1 gr. potassium metabisulphite, 1 gr. potassium bromide, 2 gr. potassium hydroxide, 120 gr. water. Each plate was developed for 5 minutes at 65° F.

² Only if an unknown was compared with standards immediately adjacent to it.

³ In the region of 4,200 Å. the intensity of the continuous spectrum was negligible. This was shown by the deflexion readings adjacent to the Rb lines and between spectra on unexposed portions of the same plate which were almost identical.

taken to avoid staining during development. The minimum deflection obtained over the line measured was recorded, and the *difference* between

SENSITIVITY OF PLATES TO RUBIDIUM LINES $\frac{4215.6}{4201.8}$

(VOLUME OF STANDARD SOLUTION USED = 0.05CCS.)

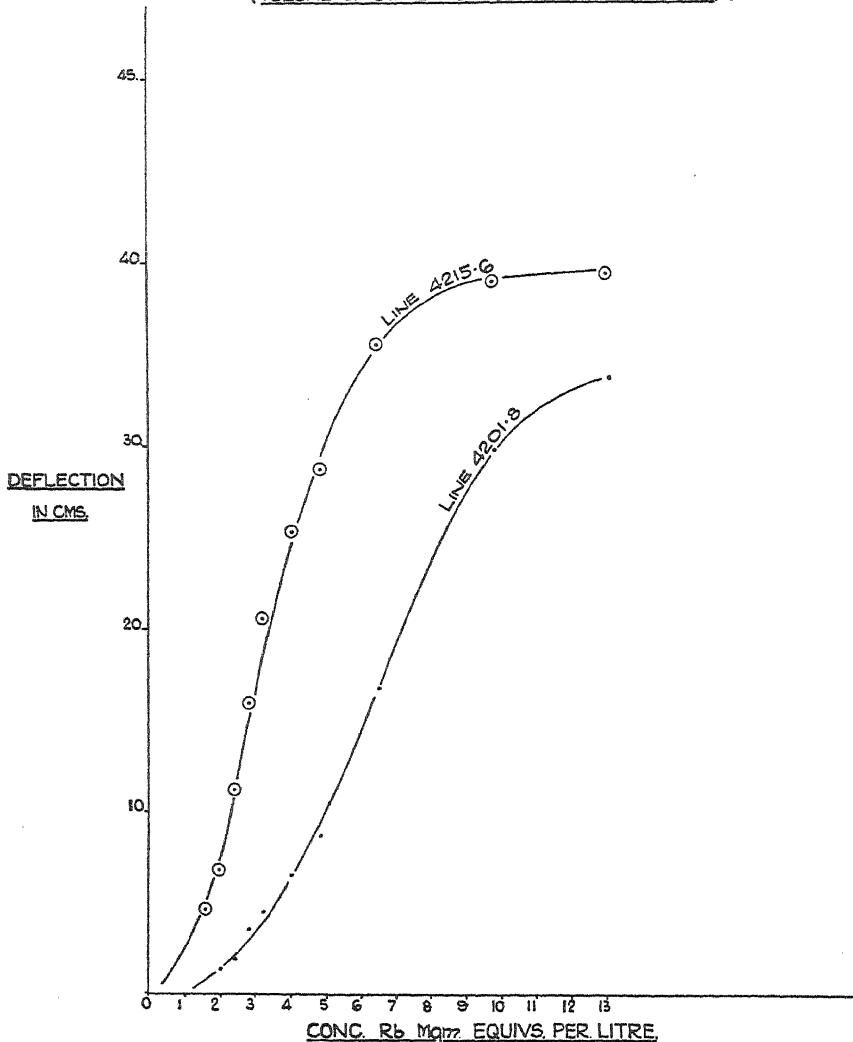


FIG. 2. Sensitivity curves of Ilford process plates to rubidium lines 4215.6 and 4201.8.

this and the mean background reading was taken as the recorded deflection. Lundegårdh (1936, p. 50) bases the calculation upon the *ratio* between the intensity of the line and the background at a fixed distance from it. This may be preferable when there is a large and very variable background

correction, but the combustion procedure used in this work eliminated such complications.¹

It is preferable to increase the sensitivity of the photometer for the weaker line (4201·8) of the doublet, and this was done in the case of Fig. 2, which shows the form of the deflection-concentration curve in the sensitive range already mentioned. The unknown results on each plate could then be obtained by comparison with the corresponding standard concentration-deflection graph. Using the dilution factor the original concentration could be calculated. Independent measurements could usually be made by use of both the 4215·6 and 4201·8 lines, but the safest duplicates are those on different plates. The mean difference between the results obtained from the 4215·6 and the 4201·8 lines, for ten estimations selected at random, 2·7 per cent.

Limitations of the method.

The combustion technique, using identical standards, produced lines which were not visibly different, although the eye can just detect 5 per cent. differences in the intensity of the lines. The microphotometer showed that variations did, however, occur. Four replicate determinations from 0·1 c.c. of 0·0024M solution yielded the deflections on a given plate of 8·7, 8·8, 8·6, and 8·5, using the line 4215·6. The maximum deviation between the quadruplicate determinations corresponded to a concentration difference of 0·00012M or a spread of 5 per cent. and a maximum difference of a single observation from the mean of 2·5 per cent.

Difficulties still remain in the use of dried, ground tissue samples. The method described when applied to solid samples does not produce precise combustion. The intensity of the lines produced by known amounts of rubidium contained in dry samples was greater than that of the lines from the same amount of rubidium free from any plant material. It was found that even sugar added to the rubidium solution increased the intensity of the lines and, since the diluted water extracts contain some soluble organic matter, the possibility that this caused an increase was investigated. Three replicate determinations of 0·1 cc. of 0·0024 M. rubidium gave deflection readings of 8·2, 8·1, and 8·0, while the same amount added to and re-extracted from potato discs and dissolved in the quantity of water originally present in the tissue gave the deflection readings 8·5, 8·5, and 8·6. These differences, due to the soluble matter in the water extracts, correspond to a difference between the mean concentrations of 0·00016 in the estimation of 0·0024 or 6·7 per cent. Though greater than the error of determination this factor can, if necessary, be eliminated by preparing the standard rubidium solutions in water extracts

¹ When the continuous spectrum cannot be avoided and slight variations in the intensity of the background occur it is convenient to determine the intensity of the background at three or four fixed distances from the line, calculate a mean background deflection, and recalculate the minimum line readings to a standard background reading (e.g. 40 cm. on the scale) and finally proceed as above.

of tissue. The presence of unusually high concentrations of potassium also increased the intensity of the lines produced by a known amount of rubidium. This effect was only of the order of 5 per cent. when potassium of strength 1·0 M. was added to a rubidium solution of concentration 0·008 M.

On pure solutions, using replicate determinations, the method is capable of an accuracy of the order of 2·5 per cent. On the potato tissue extracts used in this work the error of the mean of replicate determination need not exceed 5 per cent. This accuracy was adequate for the purpose of the experiments here described.

Application to other elements.

The technique described has obvious applications to any elements which are extractable by hot water and which give suitable lines in the flame spectra. Using the same method the other alkali metals can also be determined, and the authors have applied it to the determination of lithium and caesium. In the case of potassium the lines 4047·2 and 4044·2 were used and for amounts of 0·1 c.c. and the combustion procedure already described the sensitive range fell within the limits 0·05 M. and 0·005 M. for Ilford process plates.

EXPERIMENTAL

Comparisons between the accumulation of rubidium and of bromide by potato discs.

The comparison between the accumulation of bromide and rubidium can well be based upon the effect of three variables (partial pressure of oxygen, surface volume relations of the discs, and time) upon the absorption of these ions by potato discs. The comparison might have an even broader basis if it also included the effects of temperature and external salt concentration. This, however, will not be attempted here because unpublished investigations (concerned only with bromide absorption) have shown that at high salt concentrations or low temperature further complications, which also involve the surface volume relations of the discs, are encountered.

Experimental technique.

The apparatus and methods utilized in the experiments have been already set out by Steward (1932b). In the experiments now to be described all the essential features of this technique were adopted. Air was obtained from an automatic compressor and washed in acid and alkali. The various gas mixtures were prepared as described earlier (*loc. cit.*) and their oxygen content determined on 100 c.c. samples by absorption with yellow phosphorus in a Hempel's apparatus.

Respiration data will not be considered in this paper, since the relation between respiration and salt accumulation, in so far as it is revealed by the variables involved in these experiments, is adequately shown by the data already published by Steward (1933, 1935) on the absorption of bromide.

Analyses have been made either of the expressed sap, obtained after freezing the potato discs, or in the case of smaller samples of extracts of dried tissue. In the former case the results are expressed as mg. equivs. per litre of sap, and in the latter as mg. equivs. per 1,000 gm. of water in the living tissue. The rubidium determinations were made as already described and the bromide determinations by the method of Hibbard (1926), with the substitution of sodium hydroxide for peroxide during the ashing procedure. In both cases the methods and standard solutions were calibrated directly with the same standard rubidium bromide solution. The rubidium bromide contained only a trace of potassium.

The data of experiments, which deal with the effects of oxygen concentration, time, and disc thickness are presented in full. These experiments were all performed on tissue random-sampled from the same large stock of discs, and cut and washed in running tap-water for 24 hours. The experiments necessarily ran concurrently, so the number of separate cultures had to be reduced to the minimum; this was done to permit that close comparison between data from the different experiments which is an essential feature of the final analysis of the results. The arbitrary choice of conditions was made on the basis of evidence both published (Steward, 1933; Steward, Wright, and Berry, 1932) and unpublished.

Asprey (1937), observing that absorption of ammonium chloride during 24 hours at 25° C. by potato discs may be increased by protracted washing in running tap-water, concluded that this 'would seem to imply that it (i.e. oxygen supply) is not limiting absorption in experiments such as these to anything like the extent Steward would have us believe'. The effect of previous washing on potato discs has long been well known to the senior author. At low temperatures, comparable to those of running tap-water in the winter, potato discs lose potassium (Steward, 1937). Experiments¹ have shown that after previous periods of 1·0, 25·0, and 50 hours in running tap-water the tissue subsequently attained in 48 hours at 23° C. from 0·00075 M. KBr sap concentrations of 5·81, 13·04, and 18·94 mg. equivs. per litre, and had mean respiration rates of 0·187, 0·192, and 0·214 mg. CO₂ per gm. per hour. The effects of oxygen concentration and of washing are distinct. In the work here presented, unless otherwise stated, the comparison of the effects of time, oxygen, and disc thickness on the uptake of rubidium and bromide was made upon tissue washed under identical conditions.

The form of the oxygen pressure-respiration curve for potato discs respiring in water is known (Steward, 1933). The oxygen mixtures (4·3, 9·4, 20·9, 41·4 per cent. by volume) were so chosen that two (4·30, 9·40 per cent.) fall within the range in which oxygen concentration limits both respiration and salt uptake, and two (20·9 and 41·4 per cent.) in that range in which the changes in salt absorption and in respiration with oxygen concentration are

¹ Experiments made in 1930 in the Botany Department of the University of Leeds, in collaboration with W. E. Berry.

slight. The data obtained show (see Fig. 4) that the choice made was satisfactory.

From the work of Steward, Wright, and Berry (1932) the form of the curve which represents the relation between disc thickness and salt absorption or respiration is known. Subsequent experience has shown that the relation between disc thickness and either respiration or salt uptake can be accurately determined from data at six thicknesses which bear to each other the approximate relation of 1, 2, 3, 4, 8, 16 and cover the range from about 0·4 mm. to 6·4 mm. The mean thickness of the uniform, graded discs actually used was determined, but it is convenient to adopt instead of the absolute units a relative scale of thickness on which the value 0·121 cm. is arbitrarily designated as 6·0. To reduce the number of cultures, discs of all six thicknesses were placed in the same vessel and they were regraded according to thickness at the end of the experiment and prior to analysis.

The effect of time was determined by sampling at intervals an aliquot part of both the discs and the external liquid from the same culture vessel. In the oxygen and the time experiments only discs of thickness 3·83 on the relative scale were used. In all experiments the bath temperature was 23° C. and the tissue was sampled for analysis in the oxygen concentration and disc thickness series after 67 hours of contact with the solution. The solution in each culture vessel was two litres of 0·002 M. RbBr, which was adjusted to the temperature and aeration conditions of the experiment before the addition of the tissue.

TABLE I

Effect of Oxygen Concentration on Uptake (mg. equivs. per 1,000 gm. H₂O in tissue). Temp. 23° C. Time 67 hours.

O ₂ (% vol.)	Rb.	Br.
4·3	7·5	4·2
9·4	12·2	8·3
20·9	14·8	10·4
41·4	15·8	11·6

The oxygen concentration is that of the gas phase in equilibrium with the solution.

TABLE II

The Effect of Time on Uptake (mg. equivs. per 1,000 gm. H₂O in tissue). Temp. 23° C.

Hours.	Rb.	Br.
21·7	7·2	0·70
46·0	11·4	6·42
67·0	14·2	9·91
90·2	19·1	12·8
95·5	20·0	15·8

TABLE III

The Effect of Disc Thickness on Uptake (mg. equivs. per 1,000 gm. H₂O in tissue).
Temp. 23° C. Time 67 hours

Thickness (cm.).	Rel. thickness.	Specific surface = cm. ² /gm. fresh wt.	Rb.	Br.
0.039	1.95	60.8	18.4	13.5
0.077	3.83	22.1	14.3	10.3
0.121	6.0	15.4	11.3	6.6
0.161	7.98	10.1	9.34	5.63
0.317	15.7	6.15	7.50	2.80
0.643	31.8	3.28	6.70	1.50

EXPERIMENTAL RESULTS

The data obtained are presented in Tables I, II, III, and illustrated graphically in Figs. 3, 4, 5, and 6.

The effect of oxygen, time, and disc thickness.

The curves which represent the relation between oxygen concentration and the uptake of both bromide and rubidium are of the same form as those previously recorded by Steward, Wright, and Berry (1932) for the absorption of bromide by potato discs, and are also similar to those obtained with potato roots, discs of carrot root, and artichoke tuber by Steward, Berry, and Broyer (1936). After a brief initial period, which lasts only a few hours, the increase of rubidium and bromide concentration in the discs bears a relationship to time which is linear, at least until 100 hours. This confirms, and extends to rubidium, the results previously recorded by Steward (1932a, 1933) for bromide.

The effect of disc thickness on both rubidium and bromide concentration of the tissue is outstanding, and is represented by curves of the same kind as those previously recorded for respiration and for bromide uptake from potassium bromide solutions.

Thus far the parallelism between the absorption of rubidium and bromide is complete. Rubidium absorption, like that of bromide, is conditioned by oxygen concentration. These data supply further evidence against the claim of Lundegårdh and Burström (1933) that the absorption of cations is not determined by respiration. As for potassium so for rubidium, the conditions which produce a high rate of aerobic respiration also produce a high degree of accumulation. The pronounced effect of disc thickness implies that the rubidium absorption, like the absorption of bromide, is most intense in a thin layer of tissue at the surface of the discs—a layer previously shown to be the seat of very active aerobic respiration. The effect of time also shows that the absorption of rubidium, like that of bromide, does not proceed rapidly to an equilibrium but during a long period of intense respiration and metabolism continues unabated without any sign of approach to a condition of equilibrium.

Accordingly, all the vital processes which cause bromide accumulation, and which are dependent upon aerobic respiration, are also concerned in the accumulation of rubidium.

Figs. 3, 4, 5, 6 show, however, an obvious difference between rubidium

THE EFFECT OF OXYGEN.

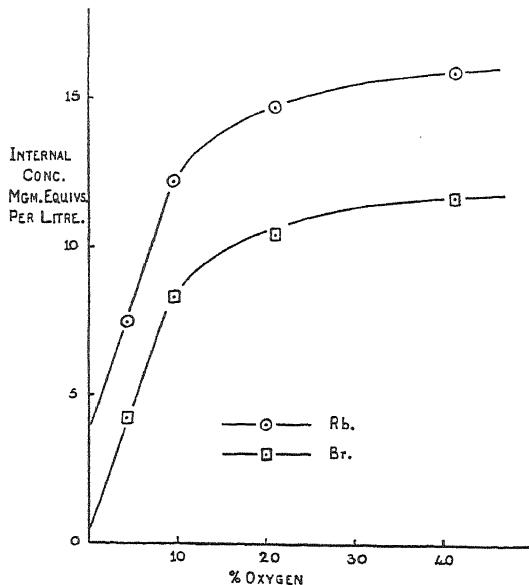


FIG. 3. The effect of oxygen concentration on the absorption of rubidium and bromide by potato discs.

and bromide absorption—the former is consistently greater than the latter. The literature abounds in cases of unequal absorption of anion and cation (Redfern, 1922; Stiles, 1924*a*, 1924*b*). Earlier work with potato discs emphasized that, after a preliminary period immediately following the immersion of the surface-dried discs, the tissue absorbed potassium and bromide in approximately stoichiometrically equivalent amounts. Stiles (1924*b*), working with storage tissues without special attention to aeration, stressed the unequal absorption of anion and cation.

The new data reported here show, however, not merely unequal absorption of anion and cation, but they also indicate that the absorption of the cation (Rb) is of two different kinds, determined by entirely different variables. The form of the time-absorption curve (Fig. 4) clearly implies that there is an initial lag period during which absorption of the bromide ion if not at zero level proceeds very much more slowly than subsequently, whereas the reverse is true for rubidium. From the data available, extrapolation of the time-absorption curve to zero time would lead to the conclusion that the tissue contained, on the whole-disc basis, a concentration of rubidium approaching

THE EFFECT OF TIME.

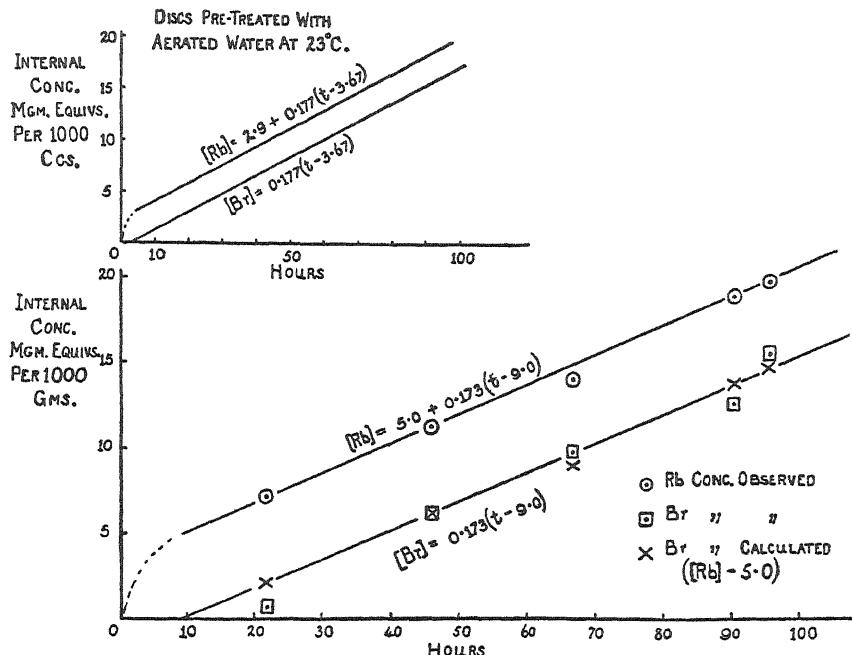


FIG. 4. The effect of time on the absorption of rubidium and bromide by potato discs.

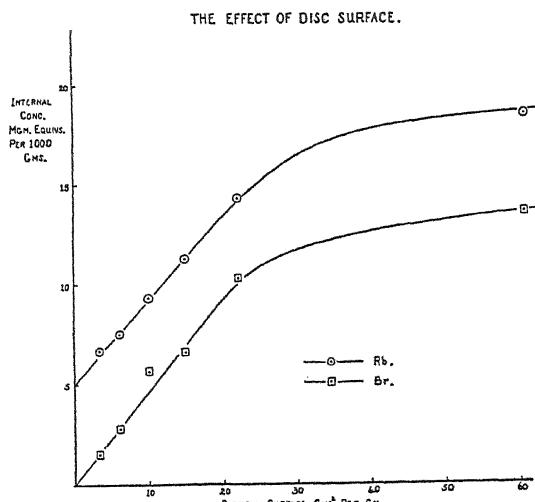


FIG. 5. The effect of disc surface on the absorption of rubidium and bromide by potato discs.

5 mg. equivs. per 1,000 gm. of water, although this element was in fact absent from the original discs. In the period of 21 hours before the first sample was taken, by which time the discs had already begun a steady absorption of both rubidium and bromide, a rapid uptake of the cation alone must have occurred.

THE EFFECT OF DISC THICKNESS.

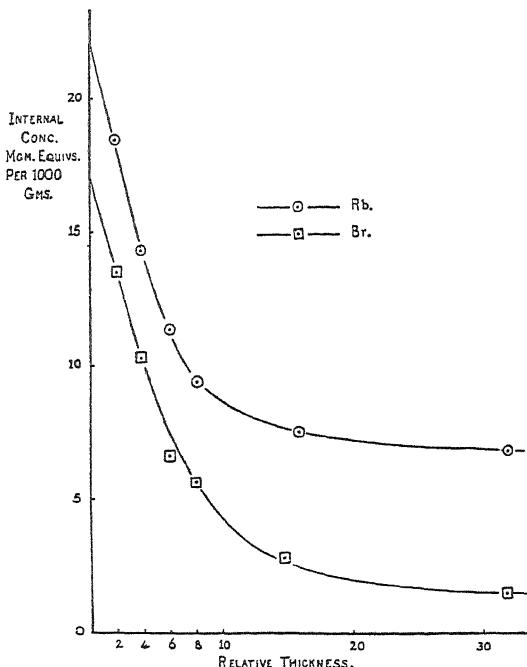


FIG. 6. The effect of disc thickness on the absorption of rubidium and bromide by potato disks.

The subsequent parallelism between the absorption of rubidium and bromide also indicates that in the period 21 hours to 96 hours the rapid uptake of rubidium unaccompanied by bromide had ceased, and that the absorption of the anion and cation was in approximately equal amounts. The difference between total rubidium and bromide content, almost constant with time, clearly approximates to the concentration of rubidium which was early and rapidly attained in the discs.

Fig. 5 shows the relation which exists between the concentration of bromide and rubidium per unit weight of tissue and the specific surface ($\text{cm.}^2/\text{gm.}$) of the discs. As they approach the zero surface ordinate these curves, like the corresponding ones for respiration obtained by Steward, Wright, and Berry (1932), may be treated as straight lines, and extrapolation to the zero surface ordinate yields limiting values for the concentration of these two ions in the tissue deep-seated within the discs. This graphical treatment eliminates the effect of the high concentrations of absorbed salt

which existed in the surface layer (see Fig. 6). As found previously by Steward (1932c) the concentration of bromide in the discs approaches zero as the specific surface decreases, indicating that the absorption was confined to a limited volume of tissue at the surface of the discs. This was not so, however, in the case of rubidium. The value at zero specific surface implies that after the period of the experiment (67 hours), rubidium had permeated the *whole disc* and attained, even in the central core of tissue in which bromide never accumulates, a concentration of approximately 5·0 mg. equivs. per 1,000 gm. of water which, it will be observed, is in excess of that in the external solution (0·002 M.). It is evident that this value agrees with the mean (5·26 mg. equivs.) of five values for the excess of rubidium concentration over bromide in the time series, and also with that concentration of rubidium which is rapidly attained in the discs.

Turning now to the effect of disc thickness (Fig. 6), extrapolation to zero thickness of the relative thickness absorption curves yields limiting values for the concentrations of the absorbed ions in the surface layers. The values obtained ($Rb = 22$; $Br = 17$) suggest that the accumulation of rubidium which occurred there, accompanied by bromide (17 mg. equivs. per 1,000 gm. water), was superimposed upon a basal concentration of rubidium (5 mg. equivs. per 1,000 gm. water) which was present throughout the whole disc.

The interpretation of the oxygen concentration-absorption curves (Fig. 3) is, therefore, clear. The basal uptake¹ of rubidium to a concentration which is again of the order of 5 mg. equivs. per 1,000 gm. water occurred independently of oxygen and therefore of any vital processes, but, superimposed upon this, the simultaneous accumulation of rubidium and bromide in approximately stoichiometrically equivalent amounts in the surface cells was controlled by oxygen concentration. The effects of oxygen supply upon the absorption of anion and cation by the surface layer of active tissue were similar—a result previously recorded for other actively absorbing systems by Hoagland and Broyer (1936), Steward (1933), and Steward, Berry, and Broyer (1936).

All the evidence, therefore, is consistent with the conclusions that *a rapid uptake of rubidium, unaccompanied by bromide, occurs in potato discs*. This uptake is complete before the tissue has attained the high rate of metabolism to which the subsequent accumulation of rubidium and of bromide is related. The preliminary and rapid uptake of rubidium differs in kind from the later absorption in the same discs, and is not comparable with the absorption of bromide and potassium which has been described in other papers of Steward (1932 to 1936). This first rapid uptake can occur at very low oxygen pressure, is not confined to those cells of the disc which are most actively metabolizing, and it quickly attains a steady value. The preliminary uptake of rubidium belongs to that heterogeneous group of absorption phenomena which were

¹ Fig. 3 shows that at zero oxygen concentration and rubidium concentration of 4 mg. equivs. per 1,000 gm. could be attained by the discs.

designated by Steward (1935) as 'induced absorption' effects. After a comparatively short period, the accumulation of rubidium and of bromide in equivalent amounts begins. This is strictly comparable to that type of absorption designated 'primary absorption' by Steward (1935), which is determined by vital processes.

From these general conclusions the effects of disc surface and time can be more closely analysed.

Surface effects in Rb and Br absorption.

The effect of the surface-volume relations of the discs upon salt absorption can be interpreted by the method outlined by Steward¹ (1932c).

The relation between R (the content of Rb or Br per unit weight of whole discs), y (the content of Rb or Br in the central core of tissue in the disc), x_s (the limiting value at the outer surface of the disc of that amount (x) of Rb or Br which, due to the activity of the surface cells, is superimposed on the basal value y) z (the depth within the disc at which the activity of the surface layer vanishes), and the dimensions of the discs (t = thickness, r = radius) is known (Steward, 1932c) and is shown diagrammatically in Fig. 7 with reference to the plane surfaces of the discs. Of these quantities t and r are known, R was experimentally determined, the values of x_s and y

TABLE IV
Constants for the Calculation of Z

Ion.	$x_s + y$.	y .	x_s .	r (cm.).
Rb.	22	5	17	1.7
Br.	17	0	17	1.7

TABLE V
The Depth, z, at which Active Accumulation of Rb and Br Vanishes

Disc thickness. No.	t (cm.).	Rb.		Br.	
		R	z (cm.).	R	z (cm.).
1.	0.039	18.4	0.046	13.5	0.048
2.	0.077	14.3	0.041	10.3	0.049
3.	0.121	11.3	0.043	6.6	0.046
4.	0.161	9.34	0.040	5.63	0.051
5.	0.317	7.50	0.043	2.80	0.047
6.	0.643	6.70	0.049	1.50	0.048
		Mean	0.044	Mean	0.048

¹ In the paper in which this method was described there are typographical errors. The argument, however, correctly leads to the result shown which can be stated in a slightly simpler form. The original account shows that the errors incurred by the only approximations which are made are small.

It may be noted here that in the well-known work of Warburg (1930) the effect of the thickness of animal tissue slices upon their metabolism was interpreted upon assumptions which clearly do not apply to tissues of plant storage organs. Recently authors have used Warburg's methods on the assumption that the discs used were less than the 'limiting thickness' in the sense of Warburg. The possible errors to which this may lead cannot be dealt with here.

are obtained from the graphs of Figs. 5 and 6, and z can be calculated. The constants have the values shown in Table IV, and Table V shows the calculated values of z for Rb and Br from data on discs which covered a very wide range of thickness.

The surface effects in rubidium and bromide absorption can, therefore, be completely interpreted by the method previously adopted for bromide on the view that the absorption of rubidium consists of two components. One

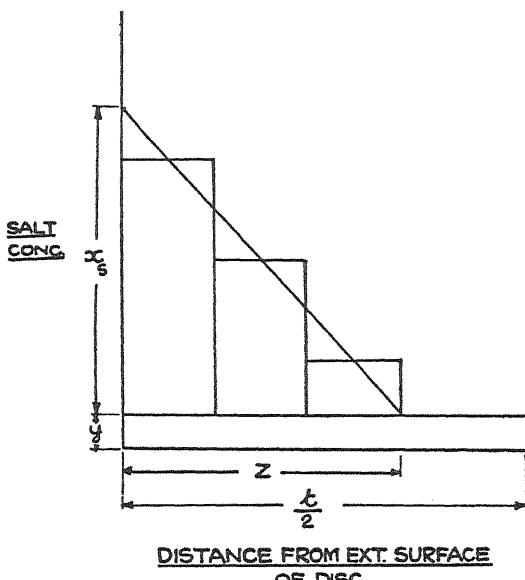


FIG. 7. Diagrammatic relation between basal uptake (y) of rubidium, accumulation in the surface cells (x_s), and depth of tissue (z) which accumulates RbBr.

of these is uniform throughout the mass of the discs and unaffected by their surface-volume relations after equilibrium is rapidly established; the other is essentially a property of the surface layers. In the surface layer those cells which are able to accumulate bromide are also able to accumulate rubidium and they do so in equivalent amounts. It must be emphasized that the depth of this layer of tissue (shown by value of z in Table V) is almost identical with the value (0.047 cm.) previously obtained by Steward (1932c) as the mean of nine independent and concordant values for the depth of actively respiring tissue at the surface of potato discs in aerated solutions at 23° C.¹

The effect of time.

The effect of time on absorption may be expressed by an equation of the

¹ Only in very strong solutions does bromide penetrate in appreciable quantity farther into the disc and then it does not accumulate beyond this level but merely attains equality of concentration (unpublished results).

form: observed rubidium concentration $[Rb] = a + b(t - c)$, in which the constants are: a , concentration of rubidium rapidly attained throughout the whole disc; c , period in hours from zero time before accumulation of rubidium and bromide begins; b , a constant of rate (conc. per unit time) the magnitude of which is determined by temperature, oxygen pressure, and external concentration.

Using the data of Table II, since the value of $a = 5.0$, the equation may be written in the form $[Rb] = 5.0 + 0.173(t - 9.0)$. For the plot of this line and agreement with the observed data, see Fig. 4.

In Fig. 4, along with the observed bromide concentration, are values calculated from the rubidium concentration on the assumption that all the rubidium in excess of the basal concentration (5.0 mg. equiv. per 1,000 gm. water) was accompanied by bromide. These figures approximate closely to the observed, and the equation $[Br] = 0.173(t - 9.0)$ can be used to describe the effect of time on the bromide uptake of the discs.

It is probable that the initial lag, during which the tissue no doubt lost some ions (see Steward, 1932a), and in which 'primary absorption' did not occur, was due to the time taken for the tissue to acquire the necessary high metabolic rate. The increase in metabolic rate is due to the transfer of discs from running tap-water below 10° C. to aerated solution at 23° C. A similar experiment on the effect of time was carried out with tubers drawn from another stock. In this experiment the discs were treated for 24 hours in aerated water at 23° C. before the addition of rubidium bromide to the solution, and the lag period was shortened thereby to 3.67 hours. The excess absorption of rubidium over bromide in this case (approximately constant from 3.5 hours to 88 hours) was 2.9 mg. equivs. per litre, and this value was attained in a period as short as 3.5 hours as shown by actual determination. At this time the bromide concentration was too low for more than mere detection. The data (inset to Fig. 4) led to the equations

$$\begin{aligned}[Rb] &= 2.9 + 0.177(t - 3.67) \\ [Br] &= 0.177(t - 3.67)\end{aligned}$$

in which it will be noted that the constant of rate is almost identical with that of the former experiment. This accords with experience, since after the steady level of metabolism is attained the respiration rate (and therefore the rate of other associated processes) of standard potato discs under the controlled conditions of these experiments is remarkably constant, even from season to season.

The nature of the absorption process.

Stiles (1924b) and Briggs (1932) respectively have considered the possibility that adsorption and ionic interchange are operative in the salt absorption by storage tissues. It remains to consider the data from this standpoint.

In so far as the progressive uptake of rubidium plus bromide ('primary

absorption') is concerned, the possibility of ionic interchange does not arise. The tissue treated with rubidium bromide showed a consistent gain in the electrical conductivity of the sap over controls in distilled water. This was also observed by Steward (1932a) and Steward and Berry (1934) in the case of discs treated with aerated potassium bromide solutions. An increment in sap conductivity usually occurs during the absorption of salts by cells active enough to increase their total salt content (Hoagland and Broyer, 1936; Steward, 1935). Nothing can be gained by again discussing the possibility that entering ions of rubidium bromide do so in exchange, not for ions pre-existing in the sap but for ions (H , HCO_3) produced in metabolism, since the difficulties which it entails have been fully explored elsewhere with reference to potassium bromide by Steward (1933 and 1935), and Steward, Berry, and Broyer (1936). The case of rubidium bromide presents no features which render the earlier conclusions inapplicable.

The possible replacement of other cations, such as potassium, by rubidium in that first or 'induced' uptake cannot be so easily dismissed. During the first period of absorption potassium and other cations are liable to leave the cells of storage tissue in excess of the amounts of rubidium absorbed (Steward, 1932a). This initial loss and subsequent reabsorption of potassium complicates a problem already beyond the accuracy of the analytical methods available.

The first rapid uptake of rubidium probably does not involve an increase of free energy because this absorption of rubidium is independent of those vital processes which represent the source of energy for cells engaged in salt accumulation, a process which commonly does occur in the direction of an increase in free energy.

The work of Devaux and later of Genevois (1928) and Genaud (1930) demonstrated that the intercellular substances of plants can bind cations and that these reactions are determined by the mass law. From the work of Bailey and Zirkle (1931) and Zirkle (1937) it is clear that the acidic substances in vacuoles may play a similar role when they react with entering bases. The adsorption of ions by tissue colloids is still another possible mechanism for ion absorption which is not dependent upon vital processes and, in fact, certain similarities between adsorption and salt uptake have long suggested to Stiles that the absorption by discs of storage tissue is analogous to an adsorption process.

The effect of concentration and the adsorption analogy.

Potato sap placed in a collodion bag which retained the colloidal constituents of the sap, e.g. protein, merely attained equality of concentration with respect to the bromide and rubidium in the external solution. There was no significant preferential absorption of rubidium either due to Donnan phenomena, or to adsorption by the colloids of the sap. The rapid uptake of

rubidium cannot therefore be a property of the substances present in the expressed sap.

Killed discs, however, did absorb rubidium in preference to bromide and they attained concentrations comparable with that reached during the first phase of the absorption of rubidium by living discs. Alcohol-killed discs

THE EFFECT OF CONCENTRATION.

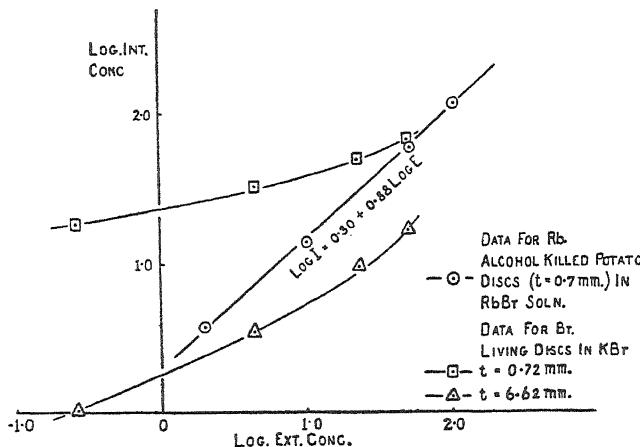


FIG. 8. The effect of concentration on the absorption of bromide by living discs and on the absorption of rubidium by alcohol-killed discs.

were exposed to rubidium bromide (0.002 M.) and after 5 hours were briefly rinsed with water. Although bromide¹ could not be estimated, this tissue contained rubidium which corresponded to a concentration of 0.003 M. even when calculated on the water content of the original-living discs. In a further experiment batches of comparable discs were weighed, killed in alcohol, transferred to water, and then exposed to rubidium bromide of known concentration until equilibrium was attained. When the discs were subsequently analysed for rubidium, after the minimum of washing to remove the solution adhering to the surface, the following results (Table VI), expressed as mg. equiv. of rubidium per litre of water in the alcohol-killed tissue, were obtained.

TABLE VI

The Effect of Concentration on Absorption of Rubidium by Alcohol-killed Discs

E = [Rb] in ext. sol.	0.002	0.010	0.050	0.100
I = [Rb] in tissue	0.0038	0.014	0.059	0.118

These data plotted on a log.-log. basis yield a straight line of the equation $\log I = 0.30 + 0.88 \log E$ (see Fig. 8). This corresponds with the relation $I = 2.0 \times E^{0.88}$, which is of the same general form as the adsorption isotherm.

¹ If equality of concentration had been attained, it is unlikely that the amount of washing used would have removed all the bromide.

Therefore the adsorption analogy might be applied to the rapid uptake of rubidium, which reaches an equilibrium value, is uniform throughout the disc, and is not dependent upon metabolism. This rubidium, however, must be loosely held since it can be displaced by protracted washing with hot water and it is expressed from living discs along with the sap. The absorption observed by Asprey (1933) in short experiments using strong not fully aerated salt solutions, by Redfern (1922), and by Mann (1924), was probably of this kind and involved properties of the tissue which are due to its chemical constitution; the active process of absorption, which is dependent upon the organization and metabolism of the living system, was apparently only involved in a minimal degree.

The adsorption analogy is not applicable to that second phase of the absorption (primary absorption) when rubidium and bromide are absorbed simultaneously and in which equilibrium is not reached even after long periods. In this process the absorbed salt contributes to the conductivity of the sap, and processes of metabolism are concerned. It is the study of this aspect of the salt uptake of storage tissues which is of most importance in the general problem of salt absorption by plants.

In Fig. 8 are two curves taken from hitherto unpublished data.¹ These relate the bromide concentration in the external solution to the internal concentration of bromide in living potato discs. For this purpose and for comparison with the earlier treatment of Stiles (1924*b*, 1936), the distribution of the bromide in the discs is assumed to be uniform. Clearly the relation between $\log I$ and $\log E$ is not linear and it involves the factor of disc thickness. Though not reported here, the experiments also yielded curves at four intermediate thicknesses which were similar to and intermediate between those of Fig. 8. Even if the deviations from the straight line are ignored, the constants of the supposed adsorption relation would vary with the thickness of the discs.

The agreement of some of the earlier experimental data with the straight line which expresses the supposed adsorption relation is only very approximate; a fact which was realized by Stiles (1924*b*), the chief advocate of the adsorption analogy. However, two such $\log I$ - $\log E$ graphs reproduced in a recent book (1936) each consist of three points, through only two of which is it possible to draw a straight line. Stiles (1924*b*) stated that he did not regard the evidence of the $\log I$ - $\log E$ graphs as 'sufficiently convincing in itself', nevertheless such evidence has been frequently cited to indicate that the absorption process is effected by concentration in a manner similar to adsorption. It seems, however, that this conclusion unduly minimizes the ability of cells to accumulate from very dilute solutions and, as the following evidence shows, it may involve some confusion between two dissimilar processes. To explain the 'primary absorption' process the adsorption analogy has no longer any value.

¹ Experiments in collaboration with W. E. Berry.

In the uptake of rubidium by alcohol-killed discs (Fig. 8) the accumulation mechanism was not involved and the absorption obtained was of the kind designated 'induced absorption'. The bromide uptake by living, aerated discs was, on the contrary, exclusively that type of salt uptake termed 'primary absorption', which is confined to surface cells and is dependent upon oxygen and metabolism. The effect of concentration upon the two processes is not the same. The absorption of rubidium in the killed discs was increased by external concentration relatively much more than the accumulation of bromide in the living cells. This contrast is most evident in thin discs in which all the cells are capable of bromide accumulation. Hence in very dilute solutions of solutes which, like rubidium, may be absorbed by *both* methods, the uptake due to the 'primary absorption' mechanism in which the metabolic processes are involved is the dominant feature. In relatively strong solutions, however, the amount of ion uptake which is attributable to the mere chemical properties of the tissue substances—in contrast to activities of the organized living cell—becomes increasingly prominent. It is the positively charged ions which are preferentially fixed by the tissue substances and therefore in strong solutions excess absorption of cation over anion may occur. Any factors which tend to suppress the accumulation ('primary absorption') mechanism, and of these deficient aeration and small specific surface of the discs are outstanding, will tend to exaggerate the apparent effect of concentration upon absorption and also upon the excess absorption of cation over anion.

The relative absorption of anion and cation.

These considerations seem to elucidate certain earlier observations of Stiles (1924*b*) and Redfern (1922*b*) in which the uptake of cation often exceeded that of the anion, although this difference tended to disappear at low external salt concentrations. Steward (1932*a*) found that, after certain complications had been eliminated, the uptake of the cation (K) by potato discs was only slightly in excess of the anion (Br.), and Steward and Berry (1934) were able to interpret the behaviour of artichoke tissue on a similar basis. Steward (1932*a*) stressed that the effect of time must be understood before the relative uptake of anion and cation can be correctly interpreted. When this is done the effects on the absorption of anion and cation due to processes which are different in kind can be discerned. Unequal absorption of anion and cation may arise from the superimposed effect of two different processes—one of which is concerned with cations only and is independent of metabolism, whereas the other is regulated by metabolism and tends to cause equal uptake of anion and cation.

However, all examples of unequal accumulation of anion and cation cannot be thus explained. Hoagland and Broyer (1936) reported cases in which positive and negative ions were actively accumulated but in unequal amounts although their absorption was clearly regulated by metabolism. These dis-

crepancies between anion and cation uptake involved the effect of time and of the composition of the tissue of the cation on metabolism and salt uptake and the explanation is not yet clear.

SUMMARY

Chemical methods for the quantitative determination of rubidium in aqueous plant extracts and saps were investigated but abandoned in favour of a spectrographic technique which is fully described.

The results of quantitative determinations of the rubidium and bromide absorbed by potato discs from solutions of rubidium bromide, and the effects upon this absorption of oxygen, of time, and of the specific surface of the discs are described. The effects of these variables upon the accumulation of bromide which were previously described have been confirmed. All the variables which affect bromide absorption affect the uptake of rubidium in a similar manner.

The absorption of rubidium is of two distinct kinds. The first phase in the absorption process is a relatively rapid uptake of rubidium *unaccompanied by bromide*. This process is unaffected by oxygen, is not confined to the surface cells, and ceases after a short time. The second phase occupies a protracted period during which both rubidium and bromide are absorbed in equivalent amounts. During this phase the absorption is confined to a few layers of cells at the surface and is determined by oxygen concentration in the manner already described for the bromide ion.

The two types of absorption are described as 'induced absorption' and as 'primary absorption' in the sense used earlier. The former is merely a property of the substances in the tissue, the latter is a process which tends to increase the free energy of the tissue, therefore demands that work should be done, and is a property of the organized living cell.

The relationships of the two types of absorption process to time have been described and they can be expressed in terms of equations.

The effect of the surface and thickness of discs upon the absorption of bromide and rubidium is interpreted quantitatively.

The distance from the surface of the discs at which 'primary absorption' ceases is the same for rubidium and bromide, and coincides with the values previously determined for the depth of the layer of tissue which exhibits enhanced respiration.

The 'induced absorption' of rubidium may be observed in discs killed by alcohol. The effect of concentration upon this process is similar to the adsorption isotherm. The effect of concentration upon the absorption of bromide by living discs is much less conspicuous and the requirements of the absorption isotherm are not rigidly met. The effect of concentration also involves the factor of surface and thickness of disc.

Unequal absorption of the bromide and rubidium arises from the superimposed effect of two distinct processes one of which causes the absorption

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of rubidium only and the other tends to cause equal uptake of rubidium and
bromide.

ACKNOWLEDGEMENTS

The purchase of rubidium salts was made out of a grant to one of us (F. C. S.) from the Dixon Fund of the University of London.

For the use of the wave-length spectrometer the authors are indebted to Professor P. M. S. Blackett, of the Physics Department, Birkbeck College.

The medium quartz spectrograph with alternative glass train was obtained with the help of a grant from the Royal Society.

We are grateful to the late Mr. Hugh Ramage for demonstrations of his methods which facilitated the preliminary stages of the work.

It is a pleasure to acknowledge the help we received from Mr. Dreblow of Messrs. Adam Hilger, Ltd.

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The Influence of Various Sources of Carbon on the Formation of Perithecia by *Melanospora destruens* Shear in the Presence of Accessory Growth Factors

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I. INTRODUCTION

IT has been previously pointed out (Asthana and Hawker, 1936, and Hawker, 1936) that the growth and fruiting of *Melanospora destruens* Shear are greatly influenced by the presence in the culture medium of certain accessory growth factors. These were obtained from a number of sources, including an extract of dried lentils and the culture media in which certain fungi had been grown. The evidence suggested that sporulation occurred when there was a suitable balance in the medium between the concentrations of nutrients, accessory growth factors, and metabolic products which retarded growth. With low concentrations of growth substance, increase in glucose percentage above 0·5 (that present in the original medium) decreased the number of perithecia formed; with higher concentrations perithecial frequency was increased as the percentage of glucose was raised above 0·5 until an optimum was reached, above which it fell sharply. Thus the concentration of glucose optimal for the development of perithecia was raised by increased concentration of growth substances.

The present paper deals with the effect of various carbon compounds on the formation of perithecia by *Melanospora* in the presence of the accessory growth factors and with a more detailed comparison of the effects of glucose and sucrose.

II. EXPERIMENTAL METHODS

The methods used were in general similar to those described in previous papers. Medium A (glucose, 5 gm.; KNO_3 , 3·5 gm.; $MgSO_4$, 0·75 gm.;

[*Annals of Botany, N.S. Vol. III, No. 10, April 1939.*]

KH_2PO_4 , 1.75 gm.; water, 1 litre; agar, 15 gm.) was again used as basal medium.

The accessory growth factors were usually added in the form of a crude lentil extract prepared after the method of Buston and Pramanik (1931), the standard dose being 0.2 per cent. (dry weight) of extract. Alternatively the source of growth substance was 'staled' medium.¹ *Botrytis cinerea* or *Rhizopus stuius* was grown for fourteen days at room temperature in half-litre flasks, each containing 50 c.c. of liquid medium A or, in the case of *Rhizopus*, medium A₁ in which nitrate was replaced by asparagin, since this fungus is unable to grow on standard medium A. The cultures were then boiled and strained through fine muslin and the liquid made up to its original volume. Since the object of the investigation was to determine the effect on perithecial formation of various concentrations of carbon compounds and growth-promoting factors, it was necessary to ensure that no complication should arise through deficiency in other nutrients. Hence although the nitrogen, phosphate, &c., of the used culture media were not completely exhausted, an excess of these was provided by again adding the quantities originally present in medium A.

For the culture of *Melanospora* the method of procedure was as follows:

(a) With *lentil extract*. To medium A, devoid of glucose, was added 0.2 per cent. (by dry weight) of lentil extract together with various amounts of the substances to be tested, viz. glucose, fructose, arabinose, galactose, sucrose, maltose, lactose, inulin, potato starch, and mannitol. In a number of more detailed experiments the concentration of lentil extract was also varied.

(b) With *staled media*. To staled media, as described above, were added various amounts of the carbon compounds listed under (a).

The addition of lentil extract necessarily involves the addition of a small amount of unknown sugars. This has been estimated as approximately 50 per cent. of the extract, so that in media containing 0.2 per cent. by dry weight of the latter 0.1 per cent. of unknown sugar is present. The concentrations of carbon compounds stated in the text below are additional to this amount. Since the nature of the sugar present as an impurity in the extract was not known, no exact allowance could be made for its presence, but as the concentrations of added carbon compounds ranged up to 20 per cent., the error due to small amounts of impurity was in general negligible.

With staled media the foregoing difficulty did not arise since the glucose originally present in the culture media was completely exhausted by *Rhizopus* and nearly so by *Botrytis*.

In addition to perithecial counts, measurements were made in some experiments of mycelial dry weight and of residual sugar in the medium. For this purpose agar cultures were unsuitable. The method adopted was to grow the

¹ As in previous papers the words 'staled medium' refer to a medium in which an organism has been grown for some time without necessarily meaning that the medium has become so 'stale' as to inhibit or seriously to depress growth.

fungus in shallow layers of medium, viz. 20 c.c. in half-litre conical flasks or in medicine bottles laid on their sides. Experience had shown that when *Melanospora* was grown in such a way that the mycelium became submerged, growth was abnormal and fruiting was inhibited. This difficulty was overcome in the manner described.

Estimations of the glucose content of staled media (after the latter had been made up to their original volume with distilled water) were made by Bertrand's method as described by Plimmer (1915). Sucrose was similarly estimated after inversion with acid and subsequent neutralization of the medium.

Perithecial frequency was estimated by the method already described (Asthana and Hawker, l.c.). Since these estimations could not be made with liquid cultures, experiments which involved the estimation of mycelial dry weight and glucose content comprised parallel series of agar and liquid media with a few exceptions. This had the further advantage that any abnormalities due to liquid culture would be more easily detected by comparison with the corresponding agar cultures. In practice a close parallelism was observed between growth and fruiting, as judged by appearance, in an agar medium and in a shallow layer of a similar liquid medium, but perithecia were usually less numerous in the latter case.

During this investigation the fertility of the strain of *M. destruens* used slowly and progressively decreased. Thus while the figures in any one table are strictly comparable, this is not necessarily true for the figures of different tables.

All cultures were incubated at 25° C. unless otherwise stated.

III. EFFECT OF VARIOUS CARBON COMPOUNDS UPON GROWTH AND FRUITING

Table I gives the perithecial frequencies of *Melanospora* cultures, seven days after inoculation, upon a series of media containing various concentrations of the carbon compounds studied. The greatest value obtained for any one compound is shown in heavier type.

On the basis of the figures shown in Table I and of accompanying cultural features of the fungus the various carbon compounds which markedly influence formation of perithecia can be arranged in a series.

At one end are glucose and fructose which give highest perithecial frequency at comparatively low concentrations, above which there is a rapid falling off in fruiting. With small amounts of these sugars perithecia are formed early and are spread fairly evenly over the plate. With higher concentrations mycelial growth is greatly increased and white aerial hyphae are formed. The perithecia develop later and tend to be aggregated on the tufts of aerial hyphae towards the edge of the plate. At still higher concentrations growth is further increased but perithecial formation is inhibited. Finally, growth is also reduced.

At the other end of the series sucrose gives no falling off in number of

TABLE I

Source of growth substance.	Source of carbon.	Percentage concentration of carbon compound supplied.						
		0.	0·5.	1·0.	2·0.	5·0.	10·0.	20·0.
Lentil extract	Glucose	3·0	7·15	3·2	few	0	0	0
	Fructose	"	8·3	3·0	"	0		
	Maltose	"	8·15	5·4	4·7			
	Arabinose	"	10·0	11·8	7·2			
	Lactose	"	8·8	11·25	10·2	9·8	7·0	
	Potato starch	"	7·7	11·1	13·1	10·2	7·0	
	Sucrose	"	2·85	3·2	5·2	9·35	9·85	few
	Galactose	"	3·5	1·6	1·0			
	Inulin	"	3·5	3·7	2·0			
	Mannitol	"	3·4	3·2	3·4	3·6	0	
Medium stale by Rhizopus	Glucose	0·65	6·95	2·45	1·4	few	0	0
	Fructose	"	7·45	3·6	1·3	"	0	0
	Maltose	"	6·6	4·95	4·0			
	Arabinose	"	9·0	14·1	10·2			
	Lactose	"	6·0	15·0	14·3	11·3	10·05	
	Sucrose	"		5·8	7·9	11·6	10·8	few
	Mannitol	"	4·05	4·1	3·8	4·0	3·55	
Medium stale by Botrytis	Glucose	1·1	2·15	1·55	few			
	Lactose	"	2·4	6·6	2·25			
	Potato starch	"	7·55	11·05	10·25	7·0	6·4	
	Sucrose	"	1·85	2·3	3·55	5·6	9·1	0
	Galactose	"	1·4	0·6	0·25			
	Inulin	"	1·75	2·7	2·0			
	Mannitol	"	2·25	3·0				

perithecia until a relatively high concentration is reached. Low concentrations of sucrose produce a sparse type of growth with few perithecia. Growth and fruiting both increase as the concentration of sucrose is raised up to 10 per cent., but are reduced by 20 per cent. sucrose.

Maltose, arabinose, lactose, and starch are intermediate in effect between glucose and sucrose. The concentrations of these compounds optimal for perithecial formation, except in the case of maltose, are between those of glucose and sucrose, while with all of them fruiting falls off more gradually as concentration is raised above the optimum than is the case with glucose.

Inulin, mannitol, and probably galactose are in a class apart since concentration of these substances within wide limits does not markedly influence growth and fruiting. Growth in every case is sparse and perithecia are fairly evenly scattered over the plate. Additions of these substances produce only a small increase in either growth or fruiting above that on media to which no source of carbon is added. Moreover, increased concentrations within wide limits do not significantly reduce sporulation. When 0·5 per cent. glucose was added to media containing various concentrations of mannitol the growth and fruiting of *Melanospora* resembled that with 0·5 per cent. glucose in the absence of mannitol. Thus the poor effect of this group of substances is not

due to any inhibitory action but is probably a result of the failure of the fungus to use them.

In an earlier paper (Hawker, I.c.) it was shown that the percentage of glucose optimal for perithecial production increased with increase in concentration of growth substance. The optimum percentage of fructose, lactose, and sucrose have been found to be similarly influenced. Thus the amount of sucrose optimal for perithecial production was increased from 2 to 10 per cent. with increase in concentration of lentil extract from 0·05 to 0·2 per cent. The percentage sucrose at which perithecial production ceases was also raised from 10 to above 20 per cent. Detailed experiments of this type were not performed with the other carbon compounds used, but observations made suggest that in all cases where concentration markedly influences fruiting of *Melanospora* the optimum percentage is similarly influenced by growth substance concentration.

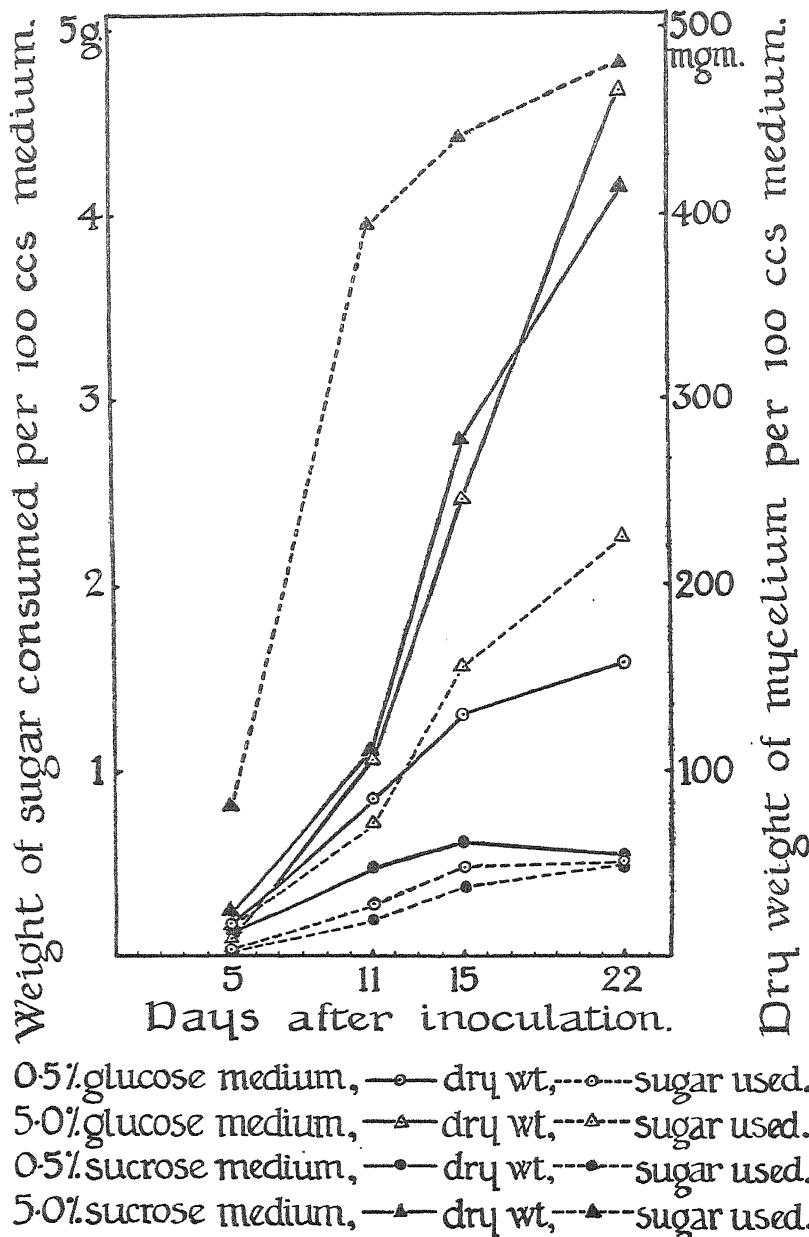
IV. COMPARISON BETWEEN EFFECTS OF GLUCOSE AND SUCROSE

An attempt was made to analyse the different effects of glucose and sucrose. The superiority at relatively high concentrations of the latter for the production of perithecia by *M. destruens* might be explained by the presence of traces of growth substances in the sample of sucrose used, the effect of a mixture of glucose and fructose in contrast to that of either alone, by differences in availability, or by a combination of these factors.

The actual rate of use of glucose and sucrose by *Melanospora* was investigated in a number of experiments. In a typical experiment the fungus was grown on media containing 0·5 per cent. glucose, 5 per cent. glucose, 0·5 per cent. sucrose, and 5 per cent. sucrose respectively. The source of growth substance was medium staled by Rhizopus since in this case no additional sugar is added with the accessory factors. The cultures were grown at room temperature. Samples were taken at intervals of 5, 11, 15, and 22 days after inoculation and the sugar content of the media, the dry weight of the mycelia, and the perithecial frequency were estimated. The results of this experiment are set out in Table II, and shown graphically in the Text-figure. Similar experiments where the cultures were incubated at 25° C. gave comparable results, but mycelial growth and utilization of sugar were naturally more rapid.

With an initial concentration of 0·5 per cent. glucose in the presence of the amount of growth substance used in this experiment, conditions are suitable for the initiation of both growth and fruiting. The glucose, however, is soon used up so that neither growth nor fruiting can proceed beyond a certain limit. (Pl. XIII, Fig. 1.)

Where the medium contains a larger initial amount of glucose (5 per cent.) growth is able to continue for a longer period but the formation of perithecia is entirely inhibited (Pl. XIII, Fig. 2). It is probable that a suitably low concentration of glucose for fruiting is not attained before the production of



Dry weight of mycelium produced and amount of sugar consumed on media containing 0.5 per cent. glucose, 5.0 per cent. glucose, 0.5 per cent. sucrose, 5.0 per cent. sucrose.

TABLE II

Sugar in original medium.	Perithecial frequency	Days after inoculation.			
		5.	11.	15.	22.
Glucose 0·5 per cent.	Dry wt. of mycelium per 100 c.c. medium	16 mg.	84 mg.	130 mg.	159 mg.
	Percentage sugar in medium	0·48	0·22	0·009	0
	Wt. of sugar consumed per 100 c.c. medium	0·02 g.	0·28 g.	0·491 g.	0·5 g.
	Perithecial frequency	0	0	0	0
Glucose 5 per cent.	Dry wt. of mycelium per 100 c.c. medium	10 mg.	106 mg.	247 mg.	467 mg.
	Percentage sugar in medium	4·84	4·28	3·44	2·75
	Wt. of sugar consumed per 100 c.c. medium	0·16 g.	0·72 g.	1·56 g.	2·25 g.
	Perithecial frequency	0	few, immature	0·75	1·25
Sucrose 0·5 per cent.	Dry wt. of mycelium per 100 c.c. medium	15 mg.	48 mg.	61 mg.	52 mg.
	Percentage reducing sugars in medium	0·05	0·02	0·02	trace
	Percentage total sugars	0·47	0·30	0·13	trace
	Wt. of sugar consumed per 100 c.c. medium	0·03 g.	0·2 g.	0·37 g.	0·5 g. (approx.)
Sucrose 5 per cent.	Perithecial frequency	0	many, immature	10·9	10·9
	Dry wt. of mycelium per 100 c.c. medium	23 mg.	110 mg.	278 mg.	415 mg.
	Percentage reducing sugars in medium	0·46	0·52	0·38	trace
	Percentage total sugars	3·80	1·04	0·56	0·19
	Wt. of sugar consumed per 100 c.c. medium	0·8 g.	3·96 g.	4·44 g.	4·81 g.

staling substances and the ageing of the mycelium have reached a point at which they become inhibitory.

When the only source of carbon in the original medium is 0·5 per cent. sucrose conditions are unfavourable both for growth and fruiting and a starved type of mycelium results with a sparse formation of perithecia. (Pl. XIII, Fig. 3. In such a medium the sucrose is inverted too slowly to give a sufficiently high concentration of reducing sugars in the medium, the amount present being always less than 0·05 per cent.

A medium containing 5 per cent. sucrose is, however, more suitable than any of the three already considered. The amount of reducing sugar present, over the first fifteen days at least, ranged round 0·4–0·5 per cent. A favourable concentration is thus maintained for a sufficiently long period to give

conditions suitable not only for the initiation but for the continuation of both growth and fruiting. (Pl. XIII, Fig. 4.)

Similar experiments with media containing 10 per cent. glucose and 10 per cent. sucrose respectively supported these conclusions, with the addition that at this concentration of sugar growth was also rather less in the glucose medium than in the corresponding sucrose medium.

A further contrast between glucose (5 per cent.) and sucrose (5 per cent.) as carbon sources is shown in Table II and the Text-figure in relation to the amount of mycelium produced per unit of sugar consumed. At the end of the experiment practically all the sucrose but less than half the glucose had been absorbed. Nevertheless the dry weight of mycelium was somewhat greater on the glucose medium. The amount of carbon consumed per unit of dry weight produced was approximately three times greater for sucrose than for glucose. Similarly, growth on a medium containing 0·5 per cent. glucose was three times as great as that on the corresponding sucrose one, although approximately the same amount of sugar was used in both cases. This uneconomic use of sucrose suggests that a large proportion of it is being converted into by-products, e.g. carbon dioxide of respiration. Experiments which are in progress and of which a fuller account will appear later support this conclusion.

In order to test the hypothesis that the favourable effect of 5 per cent sucrose upon sporulation was due to the maintenance for a long time of a favourable concentration (*c.* 0·5 per cent.) of reducing sugar, experiments were set up on media containing 0·5 per cent. glucose and carried out in such a way that the concentration was maintained approximately at that level. This was arranged either (*a*) by adding to medium A from time to time sufficient glucose to make up the deficit, or (*b*) by transferring the mycelium to fresh medium at intervals of a few days.

A typical experiment of the first kind was as follows. Cultures were set up in liquid medium A in batches containing 0·5 per cent. glucose, 5 per cent. glucose, or 5 per cent. sucrose. Three samples were withdrawn from each batch after 4, 6, 7, 8, and 11 days and used for the determination of mycelial dry weights and of residual sugar. With the data so obtained, it was possible to readjust the glucose content of certain cultures started on medium A (0·5 per cent. glucose) to its initial value. This procedure was repeated at each sampling. By this means the concentration of glucose was maintained between 0·35 and 0·5 per cent. over a period of 8–9 days. The results are set out in Table III.

Table III again illustrates the striking difference in the types of growth produced by 5 per cent. glucose and 5 per cent. sucrose. The former gives no sporulation but produces much greater mycelial dry weight in spite of the fact that an appreciable proportion is not absorbed. On the sucrose medium, all the sugar is used up, the mycelial growth is relatively small but numerous perithecia are formed. The cultures with 0·5 per cent. glucose, in which the concentration was repeatedly adjusted so as to approximate to 0·4–0·5 per

TABLE III

(For explanation see text.)

Medium.*	Days after inoculation.	Residual sugar (%)	Wt. (g.) sugar consumed per 100 c.c. medium.	Dry wt. (mg.) mycelium per 100 c.c. medium.	Perithecia.	
Glucose 0·5 per cent.	4	0·415	0·085	22	none	
	6	0·322	0·178	85	"	
	7	0·235	0·265	100	"	
	8	0·167	0·333	122	very few	
	11	none	0·500	185	few	
	20	"	0·500	206	"	
Glucose 0·5 per cent. (glucose made up to original level at each sampling)	4	0·415	0·085	22	none	
	6	0·352	0·233	84	"	
	7	0·457	0·276	131	"	
	8	0·430	0·346	154	few	
	11	0·133	0·713	246	fairly numerous	
	20	none	1·213	446	"	
Glucose 5·0 per cent.	4	4·897	0·103	20	none	
	6	4·536	0·464	160	"	
	7	4·278	0·722	252	"	
	8	3·546	1·454	305	"	
	11	2·320	2·680	487	"	
	20	1·532	3·468	720	"	
Sucrose 5·0 per cent.	4	0·457 (a)	4·613 (b)	0·387	24	none
	6	0·446	3·770	1·230	127	"
	7	0·501	3·251	1·749	209	"
	8	0·462	2·570	2·430	295	few
	11	0·132	0·908	4·092	366	numerous
	20	none	trace	5·000	385	"

* Basal medium = medium A without glucose plus 0·2 per cent. lentil extract.

(a) reducing, (b) total sugars.

cent., gave distinctly better sporulation than the similar cultures not so adjusted, so that the hypothesis outlined above was to that extent verified. They did not, however, equal the cultures on 5 per cent. sucrose in that respect.

The total amount of glucose added to those cultures in which the concentration was repeatedly adjusted to 0·5 per cent. was 1·213 per cent. Subsidiary experiments showed that an initial amount of 1·213 per cent. glucose reduced fruiting below that with 0·5 per cent. glucose (see also Table I). Moreover, in other experiments where the increments of glucose were so arranged that the total equalled 2 per cent., a few perithecia were formed in contrast to cultures with an initial amount of 2 per cent. in which fruiting was entirely inhibited under the conditions of the experiment.

The increased fruiting obtained with glucose added in instalments as compared with a high initial concentration is not due to dilution of the other ingredients of the medium or to disturbance of the mycelium. The volume

added was small and cultures which received similar quantities of sterile water showed no improvement in fruiting over those on medium A to which no additions were made.

Other experiments of a similar type, some of which were carried out in a slightly different manner or in which different initial amounts of sugars were used, gave confirmatory results. The fruiting on media receiving a large amount of glucose by instalments was always greater than that on media with the same amount given at the beginning.

In the second type of experiment discs of fine, boiled muslin were stretched over rings of cane of a size to fit loosely in a Petri dish of 9 cm. diameter. The discs were sterilized by autoclaving and were placed inside sterile Petri dishes each containing 20 c.c. of liquid medium. A small agar inoculum was placed at the centre of each muslin disc and was thus supported at the surface of the medium. The discs were lifted out at intervals of a few days and transferred to Petri dishes containing fresh medium. This involved very little, if any, disturbance of the mycelium.

The results of such an experiment are set out in Table IV. Cultures which were started on medium A and transferred to similar fresh medium at intervals of 3, 5, 6, and 7 days after inoculation were compared with others on medium A or on media containing 5 per cent. glucose or 5 per cent. sucrose which were not so transferred.

TABLE IV
(For explanation see text.)

Medium.*	Days after inoculation.	Residual sugar (%)	Dry wt. (mg.) mycelium per 100 c.c. medium.	Perithecia.
Glucose 0·5 per cent.	3	0·435	20	none
	5	0·292	76	"
	6	0·228	82	"
	7	0·140	120	"
	10	none	140	few
Glucose 0·5 per cent. (transferred to fresh medium containing 0·5 per cent. glucose at each sampling)	3	0·435	20	none
	5	0·361		"
	6	0·295		"
	7	0·280		"
	10	0·060	314	fairly numerous
Glucose 5·0 per cent.	3	4·931	16	none
	5	4·855	84	"
	6	4·500	110	"
	7	4·060	179	"
	10	3·811	344	"
Sucrose 5·0 per cent.	3	0·475 (a) 4·250 (b)	24	none
	5	0·535 4·000	61	"
	6	0·495 3·650	85	"
	7	0·487 3·231	135	"
	10	0·305 1·032	270	numerous

* Basal medium = medium A without glucose plus 0·2 per cent. lentil extract.
(a) reducing, (b) total sugar.

Repeated transference to fresh medium A gave improved fruiting compared with cultures not so transferred and with those on media with a high initial amount of glucose. As in previous experiments, the cultures with 5 per cent. sucrose produced the greatest number of perithecia. The favourable effect on fruiting of repeated transference to fresh media could not be ascribed to the presence of fresh supplies of growth substances or of nutrients other than sugar or to the prevention of accumulation of staling substances. Cultures started on 5 per cent. glucose and transferred to fresh media in which the glucose concentration equalled that in similar cultures not so transferred showed little improvement in mycelial growth and no increase in fruiting.

The above results therefore support the hypothesis that the superior value of high sucrose concentrations for perithecial production is associated with the maintenance for a relatively long period of a favourable concentration of reducing sugar. It must, however, be allowed that none of the experiments in which the concentration of glucose was maintained artificially at about the optimum value gave as good perithecial production as the 5 per cent. sucrose medium. Presumably some allowance is to be made for the fact that the glucose concentration was not maintained at the initial value but fluctuated between 0·3 and 0·5 per cent. By more frequent replenishment it might be possible to reduce the gap still further.

The data of Table V show clearly that glucose and fructose are indistinguishable in their effects upon growth and fruiting and that a mixture of the two behaves in the same manner as an equivalent quantity of either. Sucrose when inverted by hydrochloric acid is comparable in its effects with the same concentration of glucose or fructose or a mixture of both. Essentially similar results (not included in the table) were obtained by inversion of sucrose with

TABLE V

Sugar.*			Perithecial frequency after 14 days.	Growth and appearance after 14 days.
1. Glucose 0·5 per cent.	.	.	7·0	Smooth, dark. Perithecia mostly at edge.
2. Glucose 5 per cent.	.	.	0	Dark with tufts of white aerial mycelium.
3. Fructose 0·5 per cent.	.	.	7·5	as 1.
4. Fructose 5 per cent.	.	.	0	as 2.
5. Glucose 0·25 per cent. plus Fructose 0·25 per cent.	}	.	7·0	as 1.
6. Glucose 2·5 per cent. plus Fructose 2·5 per cent.		.	0	as 2.
7. Sucrose 0·5 per cent.	.	.	2·5	Thin, colourless. Perithecia scattered.
8. Sucrose 5 per cent.	.	.	10·2	Smooth, dark. Perithecia crowded.
9. Sucrose 0·5 per cent., inverted by HCl			7·15	as 1.
10. Sucrose 5 per cent., inverted by HCl			0	as 2.

* Basal medium = medium A without glucose plus 0·2 per cent. lentil extract.

invertase, but the results were complicated by the presence of active factors in the preparation used, the presence of which could be demonstrated when the latter was added to a glucose medium.

The possibility that the superiority of sucrose was due to the presence of growth-promoting substances could not be ignored. Hall and James (1933) and Hall et al. (1933) have shown that samples of sucrose contained traces of a substance which promoted yeast growth. It was found, however, that *M. destruens* made very little growth and produced no perithecia on sucrose media without the addition of growth substances, so that the amount, if any, present as impurity in the sugar was too small to account for the observed effects.

An outstanding difference between the effects of sucrose and glucose has already been pointed out (p. 462), viz. that the amount of sugar consumed per unit of dry weight produced is much less with the former. The addition of glucose by instalments, as has been pointed out, increases fruiting and to that extent approximates to the type of growth given by sucrose. With respect to efficiency in mycelial production, however, there is no such approximation. This is shown clearly by the data of mycelial production in relation to sugar consumed contained in Table III. It must be assumed, therefore, that there is an important difference in the manner in which glucose and sucrose are utilized, so that the latter is not exactly replaceable by the end products of its inversion. One may suggest that labile forms of glucose or fructose or both play a significant part in the metabolism. Further work would be necessary for the elucidation of this point.

The results described above recall those obtained by Brown (1925) in an investigation of the factors determining the growth-forms of certain strains of *Fusarium*. He found that glucose in increased concentration was more effective than starch in producing mycelial growth. Moreover, increased glucose tended to reduce, and increased starch to increase the density of sporulation. Brown pointed out that the whole of the starch in the medium might be used and yet the amount of mycelium produced was less than that on a medium containing a similar amount of glucose, and he suggested that differences in rate of respiration might account for this result. Sucrose and maltose gave similar effects to those of equal weights of glucose. In this respect the behaviour of the strains of *Fusarium* differed from that of *M. destruens*. With the former, however, the effects described relate to the production of conidia, in the latter to the perfect stage, and the requirements of these are probably different. It is also possible that the *Fusarium* strains are able to attack sucrose and maltose more rapidly than is *Melanospora*, so that conditions in a medium containing sucrose or maltose soon become similar to those on a glucose medium.

The present investigation provides a possible explanation of the well-known fact that certain commonly used natural media, such as potato extract agar and more particularly malt extract agar or oatmeal agar, are superior to synthetic media for the sporulation of many fungi. While this superiority is doubtless

associated with high content of growth substances in these natural media it may also be correlated with the presence of relatively complex carbohydrates which become available at such a rate and in such a manner as to favour sporulation. Similarly one may suggest that a synthetic medium containing sucrose, starch, or some other relatively complex carbohydrate is likely to prove more suitable for spore production than one in which the source of carbon is glucose or fructose.

V. SUMMARY

1. In the presence of a standard dose of growth substances, increased concentration of certain carbon compounds depresses perithecial formation. With glucose or fructose, the optimal concentration is low, and above that point the decline is rapid. Mycelial growth, on the other hand, continues to increase until a high concentration of the sugar is reached. With lactose, maltose, arabinose or starch, the optimum is at a higher concentration, above which the decline is more gradual. With sucrose, growth and fruiting are scanty at low concentrations, and both increase to optima at high concentrations. With inulin, mannitol, and galactose, there is little response in growth or fruiting over a wide range of concentrations.
2. The concentrations of glucose, fructose, lactose, and sucrose which are optimal for perithecial production are raised by increased addition of growth substances.
3. With low concentrations of sucrose, inversion takes place too slowly to give at any time an adequate concentration of hexose sugars in the culture medium. With higher concentrations, inversion takes place at such a rate that a favourable concentration of reducing sugars is maintained for a considerable proportion of the growing period.
4. The amount of carbon consumed per unit of dry weight produced is much greater on a medium with sucrose than on one with an equal quantity of glucose. This suggests important differences with respect to respiration.
5. The differences between the capacities of glucose and fructose media for stimulating sporulation can be partly bridged by supplying the glucose from time to time, so that a more or less even low concentration is maintained. The superior value, for sporulation, of high concentrations of sucrose lies in part in the maintenance, through enzymatic inversion, of a fairly uniform low concentration of hexose sugars. On the other hand, the high efficiency associated with growth on glucose media (see 4 above) is shown whether the glucose be added at the beginning or in instalments.
6. Mixtures of glucose and fructose are equivalent to the same total amounts of either sugar used alone. Sucrose, after inversion by hydrochloric acid or invertase, behaves similarly to corresponding amounts of glucose or fructose or of a mixture of the two. It is probable, therefore, that the difference between sucrose and glucose with regard to sporulation and growth can be fully

explained only when account is taken of the labile forms of hexose sugars which are formed as first products in inversion.

The writer wishes to express her sincere thanks to Professor W. Brown for suggesting this line of investigation, for his interest and advice during its progress, and for assistance in preparing the manuscript.

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EXPLANATION OF PLATE XIII

Illustrating Dr. Hawker's paper on 'The Influence of Various Sources of Carbon on the Formation of Perithecia by *Melanospora destruens* Shear in the Presence of Accessory Growth Factors'.

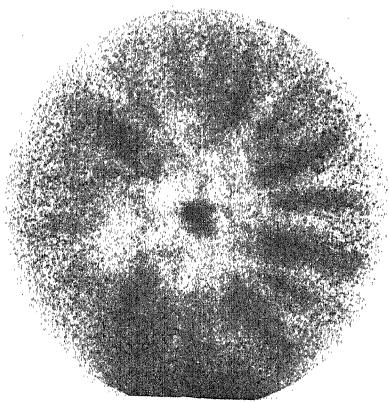
Petri dish (9 cm. diam.) cultures of *Melanospore destruens*, 14 days after inoculation, on medium A (carbohydrate varied) plus 0.2 per cent. lentil extract. Perithecia visible by hand lens in Figs. 1, 3, and 4.

Fig. 1. Medium with 0.5 per cent. glucose. Note slight darkening and perithecia more abundant towards margin.

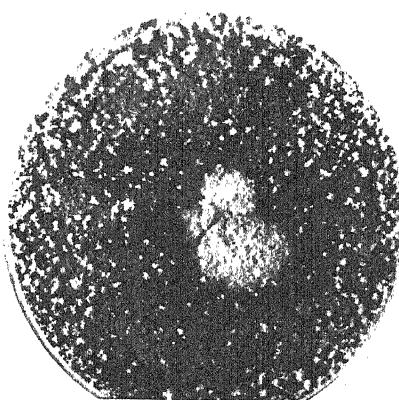
Fig. 2. Medium with 5 per cent. glucose. Intense growth with tufts of aerial mycelium, dark hyphae in the medium and no perithecia.

Fig. 3. Medium with 0.5 per cent. sucrose. Sparse growth and few perithecia.

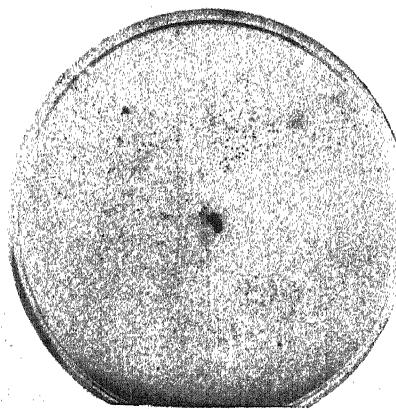
Fig. 4. Medium with 5 per cent. sucrose. Mycelium dark and perithecia distributed over plate and abundant.



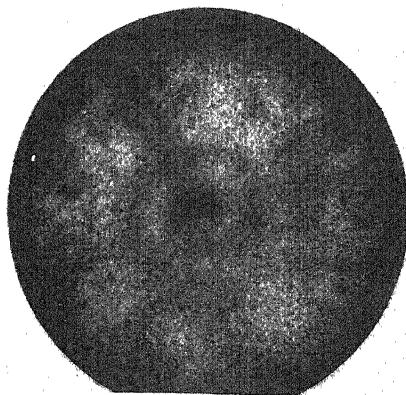
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HAWKER — NUTRITION OF MELANOSPORA.

Experimental Studies of the Relation between Carbon Assimilation and Stomatal Movement

I. Apparatus and Technique

BY

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With Plate XIV and seven Figures in the Text

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INTRODUCTION

THE role played by changes in aperture and diffusive resistance of the stomata in controlling the assimilation of carbon dioxide by the leaves of land plants has engaged the interest of plant physiologists since the work of Blackman (1895) and of Brown and Escombe (1905) established the stomata as the main paths of gaseous diffusion between the leaf tissues and the external air. In the meantime a large body of experimental data has accumulated relating changes in assimilation rate to variations in the levels of external and internal

factors. Although it has been recognized that variation in stomatal aperture must play a part in determining the rate of entry of carbon dioxide, and theoretical discussions of stomatal diffusive capacity have appeared (Brown and Escombe, 1900, 1905; Renner, 1910; Brown, 1918; Jeffreys, 1918), yet with the single exception of the work of Maskell (1928*b*) no experimental investigation has yet been published in which the changes in assimilation rate and stomatal opening were simultaneously followed in the same leaf. Indeed the tendency in assimilation studies has been to circumvent this difficulty by the use of plant material devoid of stomata, e.g. Blackman and Smith, 1911; Warburg, 1919; Harder, 1921; Wilmott, 1921; James, 1928; van den Honert, 1930; van der Paauw, 1932, so that the problem remains in the position to which Maskell advanced it, his original and powerful work not having been followed up.

Maskell measured assimilation rate gasometrically over one-hour or two-hour periods, the diffusive capacity of the stomata being estimated during each period by sampling areas of the leaf with a movable porometer cup; and he critically analysed the relations involved. Stålfelt (1935) performed short period assimilation experiments on attached leaves and measured microscopically the width of 10–20 stomatal pores by his ‘immersion method’ on each of two small pieces of leaf cut off before and after the experiment respectively. This method of stomatal measurement was subject to large errors, probably owing to the small number of stomata measured, and his data (p. 727), collected to show the variation in different parts of the leaf, yield a standard error for a mean of two such positions of about 16 per cent. Nevertheless he was able to show in a semi-quantitative manner the type of relation existing between assimilation and stomatal movement at various light intensities. He does not refer to Maskell’s work. Other experimental work on stomata and assimilation has been carried out by various authors. Iljin (1923) used a porometer cup on one-half of the leaf to measure the flow capacity of the stomata, while a short period assimilation experiment was carried out on a detached piece of leaf cut from the other half. Johansson (1926) made use of direct microscopic observation of stomatal apertures and classified them on an arbitrary scale divided into ten parts. He was only able to deal with a few stomata chosen at random. Geiger (1927) and also Johansson and Stålfelt (1928) used the infiltration method for indicating stomatal aperture. Kostytschew, Bazirina, and Tschesnokov (1928) during some of their assimilation experiments made use of a Darwin porometer. They state that ‘of course (*selbstverständlich*) the porometer measurements were not done on the same leaf as was used for the assimilation determination. Fluctuations in assimilation were found to be independent of stomatal movement. Beljakoff (1930) followed stomatal opening by infiltration with acetone, Schoder (1932) with petrol ether or xylol, and Boysen-Jensen (1929, 1932) by an infiltration method in which the whole leaf was evacuated and then the increase of pressure necessary to give half infiltration with water noted.

In recent years, Newton (1936) at this Institute carried out further investigations along the lines initiated by Maskell. Whereas the latter took advantage of the autonomous diurnal variation in stomatal aperture of the cherry laurel leaf and was thus able to work over one- or two-hour periods, Newton, using pelargonium, estimated assimilation and stomatal aperture at five-minute intervals during the opening of the stomata in response to light. In addition to experiments in which the carbon dioxide was allowed to diffuse in through the stomata from an air stream passing over the leaf surface, he carried out experiments with air actually drawn through the leaf, stomatal resistance to diffusion being thus eliminated. Newton's work was in some respects of a preliminary nature and his methods are open to certain criticisms which will be considered in the appropriate connexions. The present studies were undertaken with a view to developing the work and confirming and extending Newton's findings. Wherever the author is indebted to Newton due recognition will be made.

In the present paper the apparatus and technique employed will be described, while later communications will deal with the calibration of the resistance porometer used (Gregory and Pearse, 1934) and with the relations between stomatal movement and assimilation.

APPARATUS

i. Theoretical considerations.

In the usual gasometric method of estimating assimilation, air containing carbon dioxide is passed at a known rate through a chamber enclosing the assimilating leaf and thence to some device for determining the carbon dioxide content of the outflowing air. A parallel 'control' stream of air is usually analysed to estimate the concentration of the gas before entering the chamber. The difficulties inherent in the method are due to two opposing considerations. (1) Although the total amount of carbon dioxide assimilated may be unequivocally determined from the difference in concentration of the incoming and outgoing gas, yet the more rapid the rate of flow for a given leaf area the smaller the percentage change in composition which occurs and hence the larger, relatively, are the errors of estimation. The added practical difficulty of obtaining efficient absorption at high rates of flow requires no stressing. (2) If to circumvent these difficulties the rate of gas-flow is diminished, then the large reduction of concentration occurring renders it essential to estimate the mean concentration at which assimilation is taking place, so as to be able to correct all observed assimilation rates to a standard concentration of carbon dioxide for comparative purposes. The appropriate formula for the mean concentration is that used by Maskell (1928a), namely

$$\frac{C_1 - C_2}{\log_e C_1 - \log_e C_2},$$

where C_1 and C_2 are the initial and final values. It is obvious that to make a valid estimate of the *effective* mean concentration, all the carbon dioxide must

have been potentially available for assimilation, otherwise an over-estimate will be obtained. If the air-flow is non-turbulent, movement of carbon dioxide to the leaf from the remote parts of the chamber will depend purely on diffusion. At some distance from the leaf surface in a deep chamber, the concentration will therefore be very similar to that of the incoming gas and the diffusion gradients beyond this point will become extremely small. Hence nearly all the carbon dioxide beyond such a distance from the leaf may be regarded as potentially non-available for assimilation. This consideration has an important bearing on the design of the leaf chamber, which should obviously be made as shallow as possible so that the carbon dioxide at all levels in the chamber may be highly available for assimilation. The above considerations apply with even greater force to excessive space around the edges of the leaf, for here the diffusion 'shells' must be very much closer.

The method devised by Blackman (1895) and also used by Brown and Escombe (1905) of attaching assimilation chambers to the leaf instead of the more usual method of enclosing the leaf in a chamber has several advantages from the point of view of the present studies. It was revived by Newton (1936), and later used by Nutman (1937) for assimilation work with *Coffea arabica*. In the first place it has the advantages that the depth of the chamber can be small and that none of the experimental air supply passes round the edges of the leaf. These conditions enable valid estimates of the mean carbon dioxide concentration to be made as indicated in the preceding paragraph. Such a chamber also enables a small area of leaf to be used, which in turn means that a relatively low volume rate of flow of air can be employed without the concentration of carbon dioxide being reduced to a very low value at any point. The important consideration for the present subject of investigation is, however, that the chamber itself may be used as the porometer cup, so that the stomata whose movements are followed by this means are those concerned in the carbon dioxide assimilation measured. The question of whether stomatal movement is synchronous in different parts of the leaf does not then arise. Newton used the leaf chamber in this way in his experiments. For assimilation determinations in which the air is actually drawn *through* the leaf, such as those of Newton mentioned above and those of the author to be described later, the use of attached chambers is unavoidable.

One essential condition of assimilation experiments in which air is passed *over* the leaf surface in a chamber attached to the leaf, is that mass flow of air through the stomata shall be avoided. Newton ensured this by maintaining atmospheric pressure inside the chamber, the air-flow being produced by balanced positive and negative pressures at the opposite ends of the system. This is the method adopted here. There is, however, a further consideration, namely the existence of errors due to lateral diffusion within the leaf. Thus if the mean carbon-dioxide concentration within the chamber is greater than that over the rest of the leaf, there must be some lateral diffusion in the inter-cellular spaces. The effective area of leaf responsible for the measured assimi-

lation will therefore be larger than the area of the chamber by an unknown amount. Even if the *same* concentration is used within as outside the chamber, the carbon dioxide supply diffusing through the stomata is unlikely to be the same in the two areas owing to difference in rate of air-flow, &c. The work of both Newton and Nutman is open to this criticism, and the author has attempted to minimize such errors by surrounding the leaf chamber with a second chamber to act as a 'guard ring'. This 'guard ring' is a chamber of area equal to that of the inner chamber which it completely surrounds. Equal volumes of the same air supply are passed through both the inner chamber and the outer chamber (guard ring) while both are maintained at atmospheric pressure as above described. The air from the inner chamber only is analysed for carbon dioxide content. This method does not of course entirely remove the possibility of a lateral diffusion error, since a diffusion gradient may exist within the leaf right across the area covered by the guard ring from the inner chamber to the free portion of the leaf. The errors to be attributed to this cause will be greatest with the highest carbon dioxide concentrations used, and their magnitude will be considered in connexion with the actual experiments. For the present it may be said that they are unlikely to be important except with very high carbon dioxide concentrations, since the author's experiments with air passed *through* the leaf have indicated that once the carbon dioxide has entered the leaf it is absorbed with very high efficiency, so that the concentration at the surfaces of the assimilating cells is low. In the pelargonium leaves used the thickness between the upper and lower epidermis is about 0.13 mm., while the distance across the 'guard ring' is 6 mm. Unless, therefore, the concentration used is very high, nearly all the carbon dioxide will diffuse direct from the stomata to the neighbouring mesophyll and palisade cells rather than along lateral diffusion paths which are at least forty-six times as long.

There is one source of lateral diffusion error which is inseparable from the method of attaching the chamber to the leaf. This is due to the width of the wall or washer, which in the author's apparatus has been made as narrow (2 mm.) as seemed to be compatible with a satisfactory seal. Obviously the area of leaf under the washer can only obtain carbon dioxide by lateral diffusion, and it might appear that the most satisfactory procedure would be to add half the width of the washer all round the inner chamber in computing the area of assimilating surface. This, however, would be an over-correction, especially at small stomatal apertures, as it assumes stomatal diffusive resistance to be negligible in comparison with other resistances in the leaf (cf. Maskell, 1928*b*), and also because of the highly efficient absorption by the cells closely adjacent to the stomata as mentioned above. Probably, therefore, the most satisfactory course is to take the area of the (inner) chamber as the assimilating area in experiments with air passed over the leaf surface, recognizing this as a minimal value.

Another criticism which may be levelled against Newton's experiments

with air passing over the leaf is that he used a leaf chamber attached to the lower surface only and thus ignored the complexities introduced by the conditions obtaining at the upper surface, pelargonium being amphistomatous. The author has therefore made use of a second pair of chambers (inner and outer) which are fastened above the leaf exactly opposite to those on the lower surface. As in the case of the lower chambers, equal rates of flow of the same air supply are passed through the two upper chambers and atmospheric pressure is maintained. The air from the upper outer chamber is discarded and that from the upper inner chamber is combined for analysis with that from the lower inner chamber. It is hoped later to perform experiments in which the assimilation of the upper and lower surfaces is estimated separately, but in the present experiments the two air streams are mixed. To simplify estimations it is desirable that each surface should be assimilating under the same mean carbon dioxide concentration and therefore that the depletion of carbon dioxide should be the same in the upper and lower chambers. Since assimilation is taking place in moving air in shallow chambers, it seems most improbable that 'diffusion shells' form over the stomata and therefore interference between stomata should not occur. The assimilation might therefore be expected to be proportional to the number of stomata per unit area, and this suggests that the volume rates of air-flow through the upper and lower chambers should be in the ratio of the numbers of stomata. In the pelargonium leaves used this ratio has a mean value of about 1:14. Brown and Escombe (1905) found, however, that in all the amphistomatous leaves they investigated the assimilation by the upper surface was higher in proportion to the number of stomata, and in some cases was as much as double the assimilation per stoma found for the lower surface. They attributed this effect to the higher light intensity at the upper surface causing wider open stomata and also more rapid assimilation by the adjacent cells. A further consideration lies in the anatomical differences between the palisade tissue and spongy mesophyll which would also act in the same direction. Müller (1938) has investigated this point and found that with high illumination less assimilation occurs when leaves of *Sinapis alba* or potato are illuminated from below instead of from above. On the basis of Brown and Escombe's results it was decided to make the air supply per stoma somewhat higher in the upper chambers, and a ratio of 1:9.7 is actually used for the two rates of flow. Since only 1/10.7 of the total flow passes through the upper chambers, quite large variations in ratio of the upper to the lower stomata would cause only small errors in the estimate of the combined assimilation by the two leaf surfaces.¹ Calculations made on the assumption of equal assimilation per stoma for the two surfaces indicate that this is the case. The magnitude of such errors will be considered with the experimental results.

Dealing next with the assimilation experiments in which air is actually drawn through the leaf, it is desirable to maintain a constant rate of flow

¹ Hence the advantage of a small number of upper stomata as in the material used.

in spite of changing stomatal apertures. Newton attempted this in only one experiment, and by the difficult method of varying the head in an aspirator and hence the suction it produced. In all his other experiments with air drawn through the leaf the rate of flow varied with stomatal aperture. His method was to attach a single cup to the under surface of the leaf and draw laboratory air through the leaf under constant suction. The carbon dioxide content of the air after passage through the leaf was determined, as well as that of 'blanks' of laboratory air. In later experiments, in order to define the assimilating area, the leaf was trimmed to the outside of the cup and the cut edges were vaselined. Newton's method made it impossible to use moist air of different known carbon dioxide contents. In the author's apparatus, using the same leaf chambers as for the experiments in which air is drawn over the leaf surfaces, the air is forced into the leaf from the two outer chambers with a certain positive pressure and withdrawn via the two inner chambers with an equal negative pressure. The air therefore travels laterally through the leaf from the outer to the inner chambers, both upper and lower. It must be admitted that the distribution of the air-flow within the leaf cannot be uniform, least air flowing through the tissues under the periphery of the outer chamber and the centre of the inner chamber. Since, however, resistance to flow is small in the mesophyll compared with that through the stomata, this source of error is unlikely to be very important.

The mean pressure within the leaf tissue should be atmospheric, and mass flow of air to or from the exterior portion of the leaf should thus be mainly avoided. Unfortunately, since there is no guard ring with the apparatus used in this way, lateral diffusion errors are likely to be important except perhaps at carbon dioxide concentrations close to those of the outside air. It has therefore been found necessary in such 'through' experiments to trim off the leaf to the contours of the outer chambers and grease the cut edges.

Theoretical considerations in connexion with the use of the resistance porometer will be dealt with in the next paper of this series.

2. *The leaf chambers.*

As already mentioned there are four chambers, namely an inner and an outer chamber on both the upper and the lower surfaces of the leaf. Since these had to be accurately made of equal area a material which was both transparent and easily worked was necessary. The transparent synthetic resin 'perspex'¹ was found to fulfil the requirements. The leaf chamber apparatus was made in the workshops of the Chemical Research Laboratory, Teddington, by the kind permission of Sir Gilbert Morgan. The author is greatly indebted both to the latter and to the staff who carried out the work with consummate skill and great accuracy.

A series of circular and transverse grooves were turned or milled in the surface of a square block ($\frac{3}{8}$ in. thick) of perspex which had previously been

¹ Manufactured by Imperial Chemical Industries, Ltd.

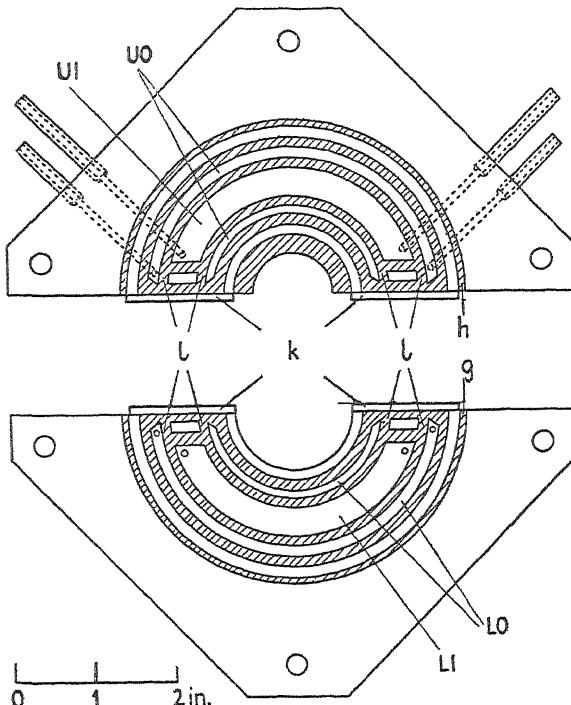


FIG. 1.

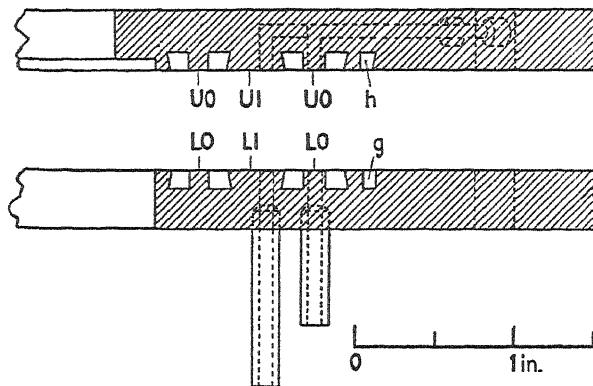


FIG. 2.

TEXT-FIGS. 1 and 2. Fig. 1. The upper and lower leaf chambers. Fig. 2. Sections of the upper and lower leaf chambers.

machined flat. These grooves are shown shaded in Text-fig. 1, and they support the basal portions of the gelatine walls or washers which form the sides of the chambers, with the exception of the grooves *g* and *h* (see below). The blocks bearing the upper and lower chambers respectively were separated by cutting the square along the diagonal, after holes had been drilled for their support on three rods on a brass frame (Pl. XIV, Figs. 2, 5). Holes ($\frac{3}{32}$ in. diam.) for the inlet and exit of gas for the four chambers were also drilled as shown in Text-figs. 1 and 2, and Pl. XIV, Figs. 4, 5, these being fitted with perspex tubes ($\frac{3}{32}$ in. bore) cemented in. After some preliminary experiments, small pieces of perspex were cemented to the edges of the chambers (Text-fig. 1 k) to support the gelatine walls at these points. The gelatine washers are cast into the grooves in the perspex by means of two brass moulds, one of which is shown in Pl. XIV, Figs. 2 and 3. The grooves in the moulds are, at the surface, the same as those in the perspex, but whereas the latter are 3 mm. deep and somewhat undercut (Text-fig. 2) the former are 2 mm. deep and slightly V-shaped. This in conjunction with a thin layer of grease in the mould ensures that the gelatine when set remains in the perspex and comes away from the mould. After casting the washers, the four small portions of wall situated at *l* (Text-fig. 1) have to be cut away down to the surface of the perspex to provide an uninterrupted passage for gas. The four assimilation chambers, the lower inner, lower outer, upper inner, and upper outer, thus obtained are denoted by LI, LO, UI, and UO respectively (Text-fig. 1). As will be seen from Text-fig. 1 and Pl. XIV, Figs. 4, 5, the outer chamber in each case completely surrounds the inner. The area at the leaf surface of each chamber is calculated to be 8.99 sq. cm. and the depth in use is actually slightly less than 2 mm. owing to compression of the washer. Without allowance for such compression the volume of each inner chamber is 1.71 c.c. and that of each outer chamber 1.67 c.c., a difference of only 2.4 per cent. due to the slightly sloping walls.

The triangular perspex block bearing the lower chambers is supported on a brass frame with an attachment for holding on a retort stand. This frame does not obscure the actual chambers which may therefore be illuminated from below if desired. Three upright bolts fixed to the frame fit accurately through the holes at the corners of the triangle (Pl. XIV, Fig. 2), and the block carrying the upper chambers slides on these bolts so that the upper and lower chambers register exactly. The leaf is placed between the upper and lower chambers, which are shaped so that, with pelargonium, the large veins near the petiole may be avoided, and the necessary pressure is applied by means of three knurled screws. Pressure is carried to the centre of the space between the two furthest screws by a brass bridging piece (Pl. XIV, Fig. 2) which prevents curvature of the perspex and consequent leaks at this point.

As previously mentioned, for assimilation experiments in which the carbon dioxide is allowed to diffuse through the stomata from air passing *over* the leaf surfaces, equal volumes from the same air supply are passed through the

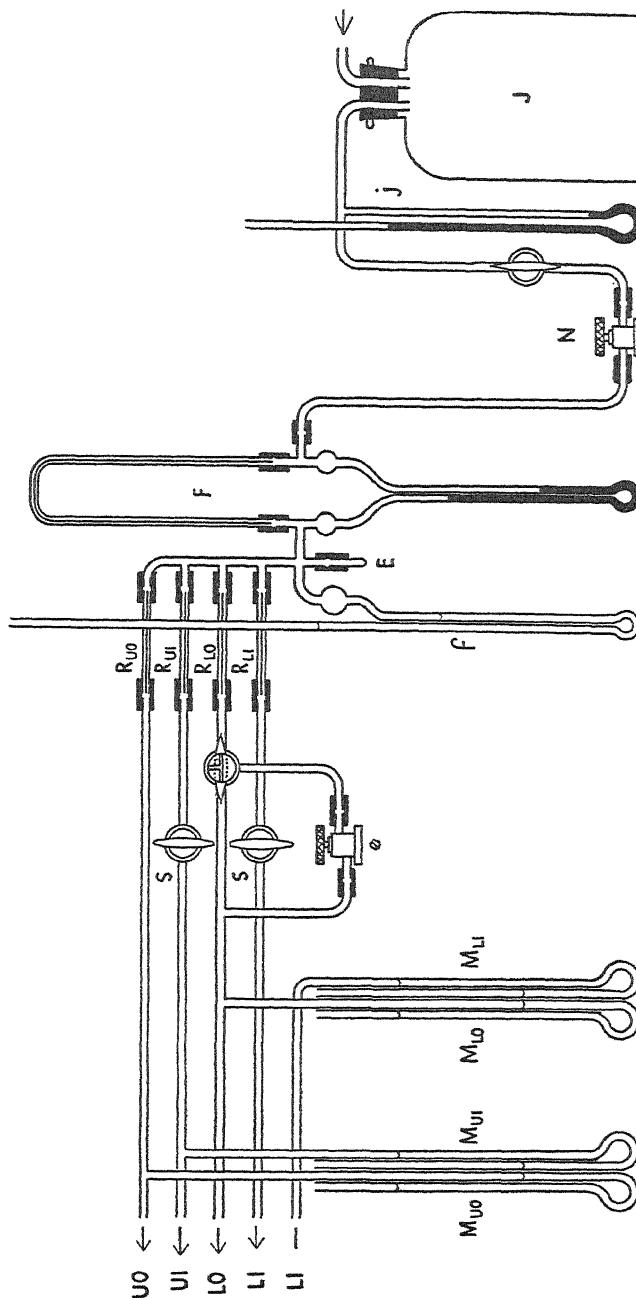
outer and inner chambers, the rate through U_1 and U_0 being $1/9.7$ of that through L_1 and L_0 . The air streams from U_1 and L_1 are mixed for analysis, while those from U_0 and L_0 , which serve as guard rings, are discarded. Such experiments will be referred to as 'over' experiments, while those in which air is actually forced *through* the leaf tissues thus eliminating stomatal diffusive resistance will be termed 'through' experiments. In the 'through' experiments air with a certain positive pressure is forced into the leaf through the stomata from U_0 and L_0 and passing laterally through the intercellular spaces is withdrawn into U_1 and L_1 with an equal negative pressure. In this use of the leaf chambers there is no 'guard ring', and it was therefore found necessary to trim off the leaf to the contours of the outer chambers and grease the cut edges in order to eliminate lateral diffusion errors. To accomplish this trimming for the part of the leaf between the two perspex blocks, a special cutting device had to be incorporated in the apparatus. Grooves g and h (Text-fig. 1) were turned in the lower and upper blocks respectively. A knife, consisting of a piece of razor-blade strip soldered to a steel carrier, fits into g , while opposite to it is a washer of specially stiff gelatine cast in h by means of an extra groove in the brass mould (Pl. XIV, Figs. 2-5). Both knife and washer project $\frac{1}{2}$ mm. above the 2 mm. leaf chamber walls, and when pressure is applied the knife, which is well greased, digs into the opposing washer thus making an efficient seal. The trimming of the rest of the leaf to the edge of L_0 is carried out by hand with a piece of razor blade.

In both 'over' and 'through' experiments the air flow is interrupted, generally for 20 seconds in the middle of each five-minute assimilation period, while a porometer reading is taken. For this purpose L_1 is used as the porometer cup, and the other three chambers are maintained at atmospheric pressure (see section 4).

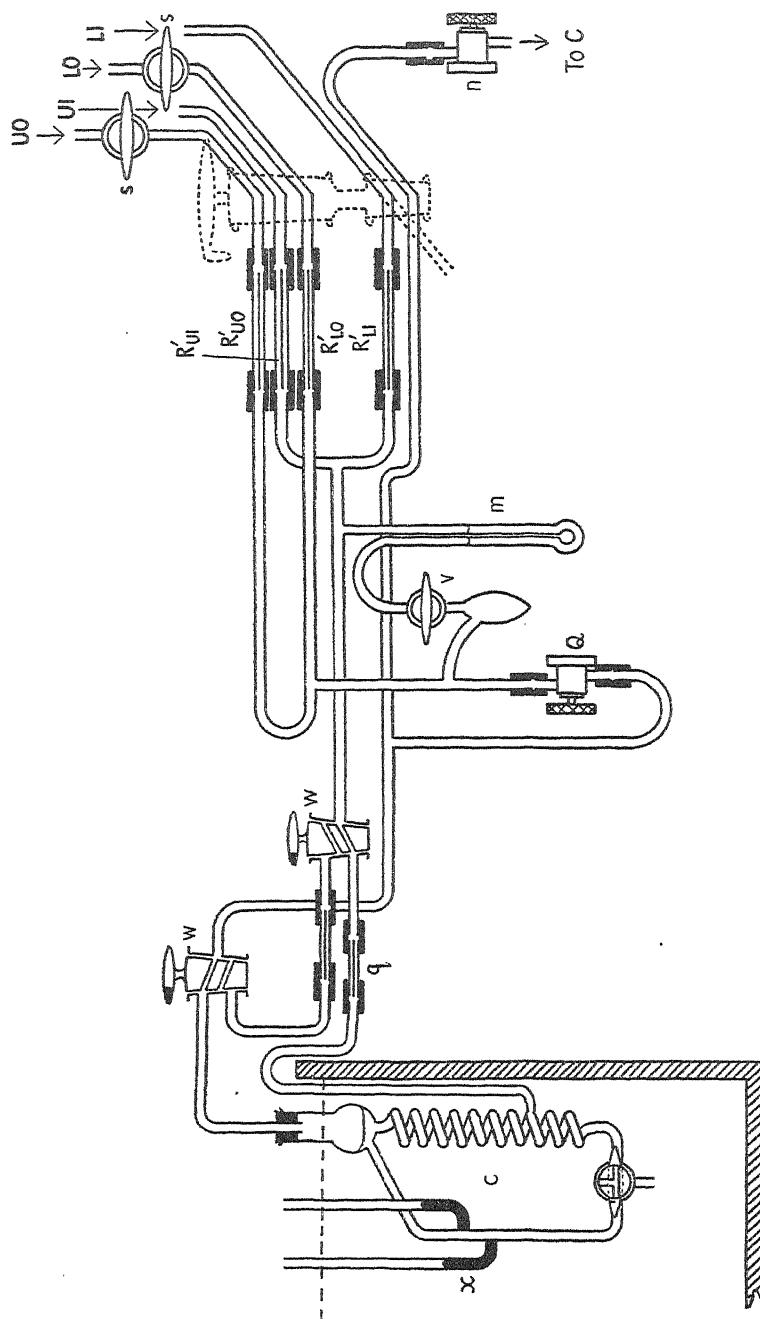
The mixture used for the gelatine washers must be carefully chosen so that it may be sufficiently soft to allow the veins to sink slightly into it and yet stiff enough not to melt or swell under the conditions of high temperature and humidity obtaining. Newton (1936) dissolved 15 per cent. of gelatine in a mixture of two parts by weight of glycerine to one part of water, such a mixture having a vapour pressure equal to the average of the atmosphere; the washers, therefore, do not dry out rapidly on keeping. In the present work the most satisfactory mixture has been 20 per cent. gelatine, and 30 per cent. for the extra rigid washer in groove h , in a glycerine-water mixture as above. To obtain gas-tight joints on to a hairy leaf such as that of pelargonium it is necessary to grease the surfaces of the washers, and a mixture of beeswax and vaseline, as recommended by Spencer (1938), has been found very suitable.

3. *The air supplies.*

The portions of the apparatus concerned with the air streams used in the assimilation determinations are shown in figs. 3 and 4. Those parts concerned in the use of the porometer are dealt with in the next section.



TEXT-FIG. 3. Apparatus concerned in the air supplies.



TEXT-FIG. 4. Apparatus concerned in the air supplies, and the conductivity cell.

a. 'Over' experiments. Air of known carbon dioxide content from a cylinder of compressed air is passed through a 10-litre jar J , the pressure in which is registered by a mercury manometer j . Its rate of flow from the jar is controlled by a needle valve N and is measured with an accurate capillary flowmeter F . The pressure at which the air is delivered by the flowmeter is measured by means of the water manometer f , and this, in conjunction with a thermometer adjacent to F , enables the rate of flow to be corrected to N.T.P. An extra T-piece E at this point enables a second air supply with separate needle valve and flowmeter (not shown) to be joined on, if it is desired to obtain variations in carbon dioxide concentration by mixing two air streams of different content. After the manometer f the air supply passes through a small bulb containing gold leaf to absorb any mercury vapour and is then split up into four streams and led to the four chambers. The relative proportions of the streams are determined by the four capillary resistances R_{UO} , R_{UI} , R_{LO} , and R_{LI} . These resistances, which are actually U-shaped (Pl. XIV, Fig. 1) are about 30 cm. long and made of relatively wide bore capillary tube which is unlikely to become partially clogged with dust or condensed moisture—a long and relatively wide capillary is used for the flowmeter F for the same reason. The resistances were calibrated so that $R_{UO} = R_{UI}$; $R_{LO} = R_{LI}$ and R_{UI} or R_{UO} is 9·7 times as high as R_{LI} or R_{LO} , thus ensuring that the volume rates of flow shall be in the proportions mentioned previously (p. 477). In 'over' experiments the four stopcocks ss , ss are all kept in the 'on' position and there is no further appreciable resistance to air flow until beyond the chambers. The four water manometers, M_{UO} , M_{UI} , M_{LO} , and M_{LI} , therefore indicate accurately the pressures in the four chambers. After passing through the chambers (not shown in Text-figs. 3 and 4) the air streams reach another set of capillary resistances R'_{UO} , R'_{UI} , R'_{LO} , and R'_{LI} . These, though not equal to the corresponding resistances R , bear the same ratios to one another. Beyond the resistances R' , suction is produced by a 'constant-pressure' aspirator C (not shown) and controlled by a needle valve n . In running an 'over' assimilation experiment, the total volume rate of flow is maintained at any desired value by means of N and F , and the positive pressure so produced is balanced by a negative pressure controlled by n so that all four manometers M show zero readings. This shows that all four chambers are at atmospheric pressure, and the resistances R , R' ensure that the rates of flow through the four chambers are in the correct ratios. After passing the resistances R' the air streams from UI and LI are joined and passed through the conductivity cell c used for carbon dioxide estimation (Newton, 1935). A short capillary resistance q is fitted before the cell to damp out pressure fluctuations due to surging therein. From the cell the air passes via n to aspirator C . A pair of three-way taps w enables the cell to be by-passed so that the absorbent need not be exhausted except during the actual experiment. The air streams from UO and LO are also joined after R'_{UO} and R'_{LO} , and they also pass via n to c . In order that the positive and negative pressures may be accurately balanced to

give atmospheric pressure in all four chambers, it is essential that the same suction be applied to all four resistances r' . The resistances of q and the cell c on the U_1 , L_1 lines have therefore to be balanced by the resistance of a needle valve Q on the U_0 , L_0 lines. That this balance has been achieved is indicated by the small water manometer m placed across from the U_1 , L_1 to the U_0 , L_0 leads, stopcock v being in the 'on' position. If m does not show a zero reading it is impossible to obtain atmospheric pressure in all the chambers as indicated by the manometers M .

b. 'Through' experiments. In the use of the apparatus for 'through' experiments, the four stopcocks $ssss$ and also the stopcock v on the manometer m are all placed in the closed position as shown (Text-figs. 3 and 4). Two small bulbs H (Text-fig. 5) containing water are fitted close to the leaf chambers to humidify the air entering U_0 and L_0 . Air passes from R_{U_0} and R_{L_0} through these bulbs to U_0 and L_0 but is prevented from proceeding to R'_{U_0} and R'_{L_0} by ss . A positive pressure results in U_0 and L_0 which is indicated by the manometers M_{U_0} and M_{L_0} . The suction on R'_{U_1} and R'_{L_1} similarly results in a negative pressure in U_1 and L_1 which is indicated by the manometers M_{U_1} and M_{L_1} . Air is therefore forced into the leaf through the stomata in U_0 and L_0 and withdrawn into U_1 and L_1 . Thence it passes to the conductivity cell for carbon dioxide estimation as in the 'over' experiments. The total rate of flow is maintained at the desired value by means of the needle valve N and flowmeter F ; and the negative pressure in U_1 and L_1 is made equal to the positive pressure in U_0 and L_0 by the adjustment of needle valve n ; each pair of manometers M is so arranged that it is only necessary to maintain level the menisci of the two inner tubes for the latter results to be achieved (see Text-fig. 3). Actually, as also in the 'over' experiments, it is only necessary to watch one pair of manometers, M_{L_1} and M_{L_0} , the other pair showing the same readings.

The manometers M may be used to detect leaks in the various chambers. Thus if a chamber is leaking seriously to the exterior, its manometer will show a zero reading even when the pressure and suction are put out of balance, with the stopcocks $ssss$ turned on as for an 'over' experiment. A leak between an inner and an outer chamber cannot be detected in this way, but will at once be apparent if $ssss$ are turned off, for then the manometers for both the chambers concerned will indicate equal pressures in the same sense instead of positive and negative.

4. *The resistance porometer.*

The principles of the resistance porometer are described in detail by Gregory and Pearse (1934), who first employed it, and need not be considered here. Those parts of the apparatus which are connected with the porometer determinations and with the change from assimilation to porometer readings and vice versa are shown in Text-fig. 5. It will be apparent that these parts lie between those of Text-fig. 3 and Text-fig. 4, but for the sake of clarity there is some slight overlapping of the diagrams.

a. 'Over' experiments. In order to change from assimilation determination to porometer readings or back again it was found when designing the apparatus that ten taps would need to be operated simultaneously. The two composite taps T and t were accordingly devised, each of which affects five ranks of tubes

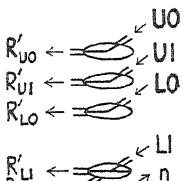


Fig. 7.

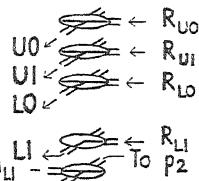


Fig. 6.

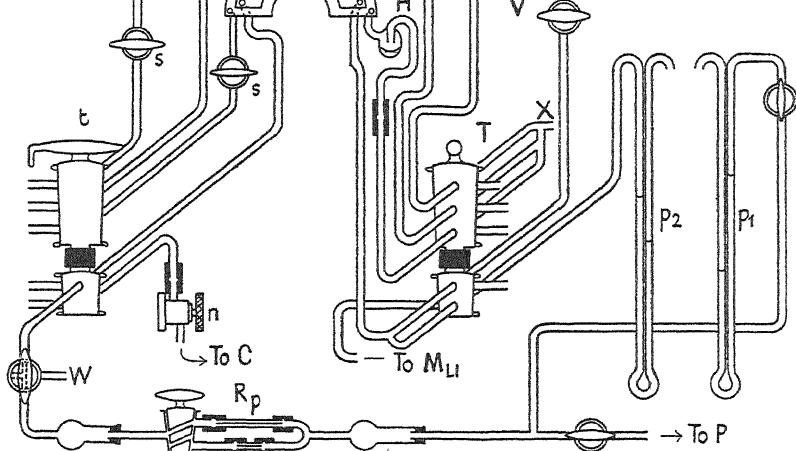


Fig. 5.

TEXT-FIGS. 5-7. Fig. 5. Apparatus concerned in the use of the resistance porometer. Fig. 6. Diagram showing position of composite tap T when set for assimilation measurement. Fig. 7. Diagram showing position of composite tap t when set for assimilation measurement.

on being turned through 90° . Each tap is made in two parts, the barrel of the upper portion being joined to that of the lower by a length of thick pressure tubing, an ebonite 'key' preventing rotation between the two barrels. Adjustable screw stops acting on one end of the handle prevent the tap from being turned too far in either direction. For assimilation measurements the two taps are set as shown in Text-figs. 6 and 7, and air therefore passes through the chambers as described in the preceding section. It should be noted that LI is in communication with the manometer M_{LI} . For porometer readings the taps are turned simultaneously in opposite directions through 90° . Tap t then shuts off all the chambers, except the 'porometer cup' LI which it places in communication with the standard capillary resistance R_p of the porometer instead of with R'_{LI} and the conductivity cell. At the lowest rank of tap t ,

the suction from aspirator c is cut off from the conductivity cell. This prevents the development in the cell of a negative pressure large enough to cause trouble with the circulation of the absorbent liquid on changing over to the measurement of assimilation. At the same time tap T places the two upper chambers and also LO in communication with a long tube x open to the exterior air. The air supply for these chambers also blows out to the exterior by the same paths. Since a supply of cylinder air is thus continuously blowing through tap T, any air taken into the chambers by the action of the porometer is uncontaminated by laboratory air. At the fourth rank of T, communication with LI is cut off and the air supply to LI blows off to the exterior via the stopcock v which must be in the 'on' position. At the fifth rank, LI is disconnected from the manometer M_{LI} , and placed in communication with the porometer manometer p_2 . The reason for thus having two separate manometers is that on changing back to assimilation measurement the meniscus in p_2 is left in approximately the correct position for the next porometer reading. This saves much time in attaining equilibrium, and in conjunction with the large area of the 'porometer cup' LI enables readings to be taken in twenty seconds.

Two standard capillary resistances, A and B, protected by calcium chloride tubes are fitted at R_p , their relative resistances being $A = 0.299$ unit, $B = 1.17$ units. By using one or the other good sensitivity may be obtained at differing stomatal resistances (cf. Gregory and Pearse, 1934), but in assimilation experiments only A can be used as a rule, since equilibrium is more quickly established with this resistance. A three-way tap w enables LI to be opened to the exterior if desired when an experiment is not actually in progress, as recommended by Knight (1915). Suction on R_p is provided by a second 'constant-pressure' aspirator P (not known) and is measured by means of the water manometer p_1 . It has been found that at the higher rates of flow the suction developed by the aspirator falls appreciably owing to the resistance to outflow of water, hence the necessity for reading p_1 . Both the porometer manometers, p_1 and p_2 , are made of precision bore tubing¹ with a guaranteed accuracy of 0.01 mm. It is, therefore, only necessary to read the level of one meniscus in each. This is carried out, using two horizontal microscopes which result in a further saving of time since their verniers need not be read until after assimilation measurement has been resumed.

b. '*Through*' experiments. In these the use of the porometer is almost the same as in the 'over' experiments. The stopcock v must be turned off to prevent the length of tube from T to s, on the LI line, from drawing in laboratory air each time a porometer reading is made, which would then be partially transferred to LI as the negative pressure redeveloped on turning T. UI draws in air from the long tube x which is full of cylinder air, so that in this case contamination with laboratory air is automatically avoided. When stomatal resistance is very high, resulting in a large reading in the manometer f (Text-fig. 3), it is desirable to send the air stream in the LO line through the by-pass

¹ Manufactured by Schott and Gen., Jena.

needle-valve resistance e (Text-fig. 3) while porometer readings are being taken. If e is correctly adjusted this maintains both f and F at approximately the readings that they have during assimilation measurement. No such extra resistance is needed on the UO line owing to the high resistance of R_{UO} .

5. *The conductivity apparatus.*

The conductivity apparatus used for measuring the carbon dioxide in the outflowing air from the leaf chambers is essentially the same as that described by Newton (1935), though as in Newton's (1936) assimilation experiments soda is used as the absorbent instead of baryta. Briefly, the air passes up the spiral of the cell c (Text-fig. 4) in bubbles, carrying some of the absorbent with it. The carbon dioxide is absorbed here, the air is drawn off at the top, and the absorbent circulates past the platinum-mercury electrodes (Text-fig. 4, x). The resistance between the two electrodes is measured by means of a very sensitive alternating current Wheatstone bridge, and the change in conductivity for a given period is proportional, over a wide range, to the carbon dioxide absorbed.

A volume of 2.60 c.c. of N/5 soda is used in the cell, being measured with an automatic pipette accurate to 0.1 per cent. The sensitivity of the apparatus, in comparison with Newton's (1935) has been improved eight-fold for the same volume of the same absorbent, and 0.001 mg. of carbon dioxide (the amount in less than 2 c.c. of ordinary air) can be detected. This improvement has been brought about by modifications in detail, notably by the use of extension coils on the bridge wire. In order to utilize the extra sensitivity of the bridge so obtained it has been necessary to pay careful attention to electrical screening of the various leads, and the thermostat bath in which the cell c is submerged has required control to within 0.002° C.; this control has been achieved by the use of a large mercury-toluene thermostat in conjunction with an Isenthal relay and by efficient stirring and lagging of the bath.

CALIBRATIONS, ETC.

i. *The flowmeter.*

Assimilation is estimated from the *difference* between the amounts of carbon dioxide entering and leaving the leaf chambers. The amount of incoming carbon dioxide is calculated from the rate of air-flow as measured by the flowmeter (F , Text-fig. 3) and any errors in this amount will be added directly to the estimation of assimilation. Small percentage errors in the measurement of air-flow will, therefore, give rise to much larger percentage errors in the difference from which assimilation is determined, especially when the latter is small. Great accuracy in the flowmeter calibration is therefore essential.

The flowmeter F was calibrated by Barr's (1934) soap bubble method, which consists in taking the time with a stop-watch for the air-flow to carry a soap film along a burette. The calibration was carried out at each of two temperatures,

$14.0-14.4^{\circ}$ C. and $23.0-23.4^{\circ}$ C., since the change in viscosity of air with temperature might be expected to have important effects over such a range. Calibrations were made at thirteen different flowmeter readings rising by 1.5 cm. intervals from 2.5 to 20.5 cm. Hg. and corresponding to rates of flow ranging from 0.446 l. per hr. to 3.710 l. per hr. Five determinations were made for every flowmeter reading at each of the two temperatures. The observed temperature effect was much smaller than theory would suggest, the mean difference between corresponding pairs of observed flow rates being only 0.5 per cent. It therefore seemed scarcely worth while to correct for temperature, since by fitting a single curve to the means of values at the two temperatures the maximum error due to this cause should be about 0.25 per cent.¹ Such a curve was accordingly calculated, the parabola of closest fit being:

$$Y = -0.0072 + 0.1789x + 0.000898x^2,$$

where Y is flow rate in litres per hour and x is flowmeter reading in cm. of mercury. It will be seen that the relation is very nearly linear and that the curve practically passes through the origin. This curve fits the data exceedingly well, the standard deviation of a single point (the mean of ten determinations each taking about a minute) being only 0.0016 l. per hr. or 0.074 per cent. of the mean rate. Over ten-minute periods it should therefore be possible to measure the air-flow to a significance of about 0.0036 l. per hr. at the mean value of 2.2 l. per hr., i.e. to about 0.16 per cent.²

A source of error which had to be taken into consideration lies in the calibration of the flowmeter when delivering air at practically atmospheric pressure while in the assimilation experiments it delivers air at a pressure of +8 cm. of water in the 'over' experiments and at anything up to +33 cm. of water in 'through' experiments. The source of error is due not to viscosity, which is almost independent of pressure, but to the existence of the factor $\left\{1 + \frac{P_1 - P_2}{2P_2}\right\}$ in the formula for viscous flow of a gas through a capillary tube which may be written

$$V = \frac{\pi r^4(P_1 - P_2)}{8\eta l} \left\{1 + \frac{P_1 - P_2}{2P_2}\right\},$$

where V is volume per unit time measured at pressure P_2 , $P_1 - P_2$ is the pressure drop along the capillary, η is the viscosity, r the radius, and l the length. Calculation shows that the error due to this cause amounts to only 0.1 per cent. for the 'over' experiments and to not more than 0.3 per cent. in the 'through' experiments. The above mentioned factor accounts for the quadratic term in the calibration curve of the flowmeter, the amount of curvature being almost exactly that demanded by theory.

¹ Assimilation experiments have been performed at laboratory temperatures generally between 18° C. and 22° C., but occasionally as low as 15° C. or as high as 23° C.

² Such a deviation would be exceeded by chance only once in twenty trials.

2. The capillary resistances.

These resistances (Text-figs. 3 and 4, R,R') were also calibrated in the first instance by means of Barr's (1934) method, air being drawn through them at rates comparable with those to be used in the assimilation experiments by a constant pressure aspirator. The resistances were adjusted by grinding the ends so that under the same head the flows obtained were in the ratios desired (see p. 481). Absence of turbulence was shown by doubling the head in the aspirator when the flow was found to increase proportionately. The greatest difference found between two resistances supposed to be equal was that between R'_{U1} and R'_{U0} which amounted to 0.56 per cent. Since these only carry $1/10.7$ of the total flow they are relatively unimportant. In none of the other pairs of resistances was the difference greater than 0.16 per cent., and the total flow through the four 'outer' resistances differed from that through the 'inner' by only 0.13 per cent. In 'over' experiments, of the total flow measured only the air from the inner chambers is analysed, and therefore accurate division of the air streams between inner and outer chambers is important. The ratios of the resistances were finally checked by arranging them in sets of four as in a Wheatstone bridge with a water manometer across (Chinoy, 1935) and passing air at 2.25 l. per hr. This showed that the maximum pressure difference to be expected between the upper and lower chambers was 0.5 mm. of water, and this has been confirmed in the actual use of the apparatus in assimilation experiments.

3. Flow through the leaf chambers.

It is desirable that the air should flow evenly over the leaf surface without turbulence and that there should be no 'dead areas'. The leaf chambers were tested in the first instance, with a glass plate in place of a leaf, by the Aeronautics Department of the Imperial College, to whom the author is indebted for the method. Later, the tests were repeated with a leaf actually in position. Small crystals of potassium permanganate were embedded in the gelatine washers, and water was passed through the chambers at a measured rate of flow which was increased until the stream lines made visible by the permanganate solution broke up in turbulence. No signs of turbulence or dead areas were found at rates of water flow corresponding to at least 10 l. per hr. of air. Since in the assimilation experiments the rate of flow through any one chamber does not exceed 2 l. per hr. this is entirely satisfactory.

4. Volumes and time lags.

A certain time must necessarily elapse between the passing of the air through the leaf chambers when assimilation takes place and the passage of that air into the conductivity cell. For this reason the volumes of the tubes between the leaf chambers and the cell were made as small as possible. A further lag will be due to the time necessary for circulation of the absorbent in the cell.

The volumes of the tubes in question were measured by a method of sharing

pressures with a vessel of known volume. The volume from UI to the top of the cell was found to be 5·2 c.c. and the corresponding volume from LI was 6·6 c.c., the common path being shared in the ratio of the rates of flow. For a total rate of flow of 2 l. per hr. this gives a weighted mean time lag of 21 sec., the lags for the upper and lower chambers respectively being 99 and 13 sec. When only the lower chambers are used, as with a hypostomatous leaf such as that of begonia, the lag for 2 l. per hr. is 12 sec. A test with Indian ink of the time for circulation in the cell showed that for the absorbing solution to travel from the point of entrance of the air round to the bottom of the electrode chamber took 30 sec. and that this was almost independent of rate of air-flow. At 2 l. per hr. with both lower and upper chambers there is therefore a total time lag of about 50 sec., and bridge readings should be made 50 sec. late to allow for this. This was not done in some of the earlier experiments, in which therefore a correction must be made in calculating the assimilation for the first five minutes after illuminating the leaf.

5. The conductivity cell.

The calibration of the conductivity cell in terms of air from a cylinder was carried out at each of seven different rates of air flow on six occasions, i.e. with six separate fillings of the cell, each value being obtained from a run of 22 or 33 minutes. Analysis of variance was applied to the 42 values of the change in conductivity per unit volume of air b/q . These values were found not to be constant but to fall with increasing rate of flow q indicating a fall in efficiency of absorption. The variance between rates of flow was highly significant showing that the curve relating change in conductivity per minute b to rate of flow q is not a straight line. On the other hand the variance between fillings was not significant, indicating that a single mean curve for all fillings would suffice and that in assimilation experiments a blank determination for each filling would be unnecessary. The standard deviation of a single 'observation' taken within rates but over all fillings was 1·8 per cent. of the mean, each 'observation' being a regression coefficient b fitted to 12 points divided by the estimated flow q . Plotting $\log b$ against $\log q$ was found to give a straight line up to $q = 2$ l. per hr., and a linear regression of $\log_{10} b$ on $\log_{10} q$ was accordingly fitted. This was found to be

$$\log_{10} b = 0.9718 \log_{10} q - 1.8098,$$

giving

$$b = 0.01550q^{0.9718},$$

where b is in mhos $\times 10^{-7}$ per minute and q is in c.c. per hr. at N.T.P. Calculating from the equation b/q for each observed q , and taking the deviations b/q observed minus b/q calculated, a standard deviation of 1·8 per cent. of the mean was again obtained. This agreement with the result obtained by analysis of variance when 'between rates' variance was eliminated indicated that the curve had also eliminated this source of variation.

In order to convert such calibration into terms of carbon dioxide, it is necessary to know the concentration of the gas in the cylinder air used. Estimations were carried out by absorption in 0.2 N soda, contained in two spiral 'bubblers' in series, and analysis in a Van Slyke apparatus. The volumes of air passed through the 'bubblers' (6 litres at N.T.P.) were measured by means of the flowmeter F. The carbon dioxide content of the air used in calibration was 0.626 mg. per l. at N.T.P. or 0.0313 per cent. by volume. Knowing q from the flowmeter reading, the weight q' mg. of carbon dioxide per minute passing into the leaf chamber can therefore be calculated. The corresponding quantity of carbon dioxide passing into the cell is given by $b' \times q'/b$ where b is calculated from the above equation and b' is the change in conductivity per minute observed. In the actual assimilation experiments with low carbon dioxide another cylinder of air was used having a carbon dioxide content of 0.596 mg. per l. The weight passing into the leaf chamber is therefore $q' \times 0.596 \div 0.626$ mg. per min. The same conductivity cell has also been successfully used for air of medium carbon dioxide content (3.37 mg./l. or 0.169 per cent.), but for high concentrations a larger cell is necessary.

The above-mentioned standard deviation of 1.8 per cent. for b/q is a measure of the *accuracy* of the method and includes errors in filling the cell, flowmeter errors, and so forth. The *sensitivity* of the method is given by the smallest quantity of carbon dioxide that can be detected as passing into the cell. With 2.60 c.c. of 0.2N soda the initial sensitivity is found to be 1.08×10^{-3} mg.¹ CO₂ and the theoretical sensitivity has been estimated at 0.91×10^{-3} which is in good agreement. The sensitivity improves as the conductivity falls with the absorption of carbon dioxide. The sensitivity of the apparatus sets a limit to the accuracy of the assimilation measurements. Thus if 2 l. per hr. of air containing 0.596 mg. per l. of carbon dioxide is passing through the inner chambers in an 'over' experiment, and the conductivity method is sensitive to 1.1×10^{-3} mg. of carbon dioxide, the amount in 1.8 c.c. of the cylinder air, then for a single 4 min. 40 sec. assimilation period the accuracy could only be 1.2 per cent. even if all the carbon dioxide was assimilated. Actually the assimilation found in such an experiment generally varies from about 15 to 70 per cent. of the carbon dioxide, under which conditions the corresponding figures for the accuracy would be 8 per cent. to 1.7 per cent. It must be emphasized that these figures are for a single assimilation period of 4 min. 40 sec., and that the accuracy will be much greater for a curve fitted to values from a number of such periods.

6. Light intensity.

The source of light is a 1,000 watt gas-filled lamp, the filament being about one foot distant from the leaf chambers. A white reflector is fitted above the

¹ This value is approximately halved if 0.1N soda is used, but the period for which the apparatus can be run without refilling the cell is then somewhat curtailed. With 0.2N soda this period is about 9½ hours with ordinary air passing through the cell at 2 l./hr. and no assimilation. The corresponding time for 0.1N soda is about 1½ hours.

lamp (Pl. XIV, Fig. 1) and a cooling screen of running water with a second screen of concentrated potash-alum solution cut off the greater part of the radiant heat. The light intensity under the perspex of the leaf chamber was measured with a Weston Photronic Cell and meter and was found to be 2,450 foot-candles. When a layer of gelatine of the thickness of the washers (4 mm.) was added and greased, the light intensity fell only to 2,330 foot-candles. The portions of leaf under the washers are therefore almost as highly illuminated as the remainder.

The above value of 2,450 f.c. is the maximum light intensity as used where light is required to be 'in excess'. For experiments on light limitation of photosynthesis it can be reduced by the use of neutral screens.

7. Temperature in the leaf chambers.

Newton (1936) used a platinum resistance thermometer in his leaf chamber, but this was not desirable in the present apparatus as it would interfere with the smoothness and transparency of the chamber. In any case only a thermo-couple in the leaf to give the tissue temperature would be really satisfactory. A thermometer with its bulb encased in a perspex sheath adjacent to the leaf chambers is accordingly used to give some indication of the temperature in the chambers. Comparison of the temperature as shown by this thermometer with that shown by another propped under a leaf between the chambers shows that it registers about 3·5° C. too low, the differences ranging from 2·6° to 3·9° C.

In order to maintain the temperature as constant as possible, the lamp is left on throughout an experiment and when darkness is desired a sheet of $\frac{1}{32}$ in. ebonite is inserted between the water screen and alum bath. The temperatures in the leaf chambers range from about 25° to 30° C. without the ebonite, so that temperature is unlikely to be 'limiting' assimilation. With the ebonite the temperatures are some 2° to 4° C. lower.

8. The porometer resistances.

The two resistances R_p , A, and B were calibrated against a standard resistance and found to have the relative values $A = 0\cdot299$ units, $B = 1\cdot17$ units, as mentioned above. They were also calibrated in absolute terms by passing air through them at measured pressures (f) and rates of flow (F). Absence of turbulence was shown by doubling the pressure when the flow increased proportionately. It was therefore permissible to make use of the formula given on p. 486 to evaluate the resistances in terms of $\frac{l}{r^4} \text{ cm.}^{-3}$. Thus A was found to have a resistance equal to that of a tube of circular cross section of the dimensions $\frac{l}{r^4} = 1\cdot130 \times 10^8 \text{ cm.}^{-3}$, the corresponding value for B being $4\cdot394 \times 10^8 \text{ cm.}^{-3}$. Hence one 'unit' is equivalent to $3\cdot77 \times 10^8 \text{ cm.}^{-3}$.

TECHNIQUE OF AN EXPERIMENT

a. 'Over' experiments.

The leaf, either attached to a potted plant or provided with a water supply by the method of Gregory (1938), is placed in the dark for a suitable period in order that the stomata may close. It is then supported between the upper and lower leaf chambers so that they are fairly near to the periphery, very large veins being thus avoided. The upper chambers are pushed down until their gelatine walls press the leaf lightly against the lower washers or, if a 'through' experiment is to be performed later, until the washer in h (Text-fig. 1) presses it lightly against the knife. The screws are then tightened in order, $\frac{1}{4}$ or $\frac{1}{8}$ turn at a time, until all the chambers are free of leaks as shown by the manometers M (see p. 482). The leaf is kept in the dark until the beginning of the experiment, time being allowed for recovery from the 'shock' of setting up. When the conductivity cell has been filled and its 'wet resistance' found using carbon dioxide-free air (see Newton, 1935), then with the light 'on' and the ebonite sheet in position and air flowing at atmospheric pressure and at the desired rate through the chambers, the taps w (Text-fig. 4) may be turned so that the air passes through the cell and readings begin. It is usual to take readings for several five-minute periods with the leaf in darkness at the beginning and end of each experiment in order to estimate the respiration rate. Readings of the bridge are taken at times 0, 5, 10, &c., minutes. If the time lag is 50 sec. the light should be uncovered at say, 9 min. 10 sec. In order that the porometer reading may fall precisely in the middle of the actual five-minute assimilation period, which may be, for example, from 9 min. 10 sec. to 14 min. 10 sec., the air-flow must be interrupted from 11 min. 20 sec. to 11 min. 40 sec., the final adjustment of the reading microscope to the meniscus of p_2 (Text-fig. 5) being effected at 11 min. 40 sec. and the taps t , t' immediately turned. If an assistant is available to take bridge readings it is desirable to take these not only at, say, 10 and 15 minutes, but also at 11 min. 50 sec. and 13 min. 10 sec., the interruption for the porometer reading then lasting from 11 min. 55 sec. to 12 min. 15 sec. The assimilation rate can then be calculated from the two 1 min. 50 sec. periods, thus eliminating the effect of any air which has passed through the leaf into L_1 during the porometer reading and also allowing a few seconds for the re-establishment of diffusion gradients in the leaf to begin. When assimilation rate is steady, the rate for the first 1 min. 50 sec. period may be compared with that for the second, and a further estimate of the errors arising from the use of the porometer may be made by means of occasional five-minute periods without porometer readings. The magnitude of such errors will be considered in connexion with the experimental results.

In addition to the regular readings of the porometer manometers and the bridge, the manometer f is read occasionally and the temperatures at the flowmeter F and in the perspex sheath by the leaf chamber are noted periodically. F is maintained at a constant reading and the manometers M at zero readings by adjustment of the needle valves N , n .

b. 'Through' experiments.

These are generally carried out on the same leaf after an 'over' experiment, sometimes the next day. In setting up the leaf chambers both the washer in *h* and the knife are well greased. When a 'through' experiment is to be performed the outer part of the leaf is trimmed off close to the leaf chamber with a piece of razor blade and the cut edge is heavily greased. At least an hour is allowed for recovery from 'shock', during which time the leaf is darkened. Since the latter is without a water supply it is essential to moisten the air passing into *UO* and *LO* by means of the humidifying bulbs *H* (Text-fig. 5), and air should not be passed through the leaf until shortly before readings are to be begun.

The method of controlling the air streams in 'through' experiments has already been described (p. 482). The pressure indicated by the manometer *f* changes continually with stomatal resistance and must therefore be read at regular intervals. If the rate of flow is to be altered, this is done by a definite amount at the beginning of a five-minute period. The use of the porometer is the same as in 'over' experiments, but since air passes through the leaf into *LI* both in assimilation and porometer determinations, and since there are no diffusion gradients to re-establish it is unnecessary to take bridge readings except at the beginning and end of each five-minute period. The porometer readings may therefore be taken exactly in the middle of the actual five-minute assimilation periods as above described, and the assimilation rate is calculated from the full 4 min. 40 sec.

SUMMARY

1. Some theoretical considerations are put forward on the design of an apparatus for studying the relation of carbon dioxide assimilation and stomatal movement.
2. An apparatus is described and illustrated which enables assimilation to be measured at short intervals with considerable accuracy, and stomatal movement to be followed simultaneously on the same portion of the leaf. The technique employed in using the apparatus is described in detail.
3. With this apparatus experiments of two types have been carried out, namely 'over' and 'through' experiments. In the first air is passed *over* the leaf surfaces and carbon dioxide passes into the leaf by diffusion through the stomata. In the second air is forced *through* the leaf so that diffusive control by the stomata is eliminated.
4. Two pairs of transparent leaf chambers are attached above and below the leaf in such a manner that the upper and lower chambers exactly register. Each pair consists of an inner chamber surrounded by an outer chamber of equal area.
5. In 'over' experiments air streams of the same known carbon dioxide content are passed through all the chambers at flow rates approximately proportional to the numbers of stomata on the surfaces enclosed. The ratios

of the flow rates are controlled by fixed capillary resistances, and atmospheric pressure is maintained in the chambers during flow by a method of balanced pressure and suction. Assimilation is measured by a conductivity method on the gas from the inner chambers only, the outer chambers serving as 'guard rings' to prevent lateral diffusion of carbon dioxide during assimilation.

6. In 'through' experiments air is forced into the leaf through the stomata from the outer chambers with a certain positive pressure and withdrawn into the inner chambers with an equal negative pressure, thus travelling laterally through the intercellular spaces; the mean pressure in the leaf meanwhile being maintained at atmospheric. From the inner chambers it passes to the conductivity cell for analysis. By keeping the rate of flow constant any stomatal control of carbon dioxide supply to the intercellular spaces is eliminated.

7. In both 'over' and 'through' experiments five-minute readings of assimilation are taken during stomatal opening. Stomatal aperture is estimated by the resistance porometer method (Gregory and Pearse, 1934), the lower inner chamber being used as a porometer cup. The stomata concerned are thus identical with those responsible for the assimilation measured. In the middle of each five-minute assimilation period, air-flow is interrupted for twenty seconds while a porometer reading is made. The change over from assimilation to porometer and vice versa is effected by turning simultaneously two 'composite' taps, each of which operates on five flow lines.

8. The calibration of the various components of the apparatus is fully described and the errors involved in their use are considered in detail.

9. The total air-flow may be measured significantly to within 0.16 per cent. for ten minutes flow. In 'over' experiments it is divided equally between the inner and outer chambers with an accuracy of the order of 0.1 per cent., and the greatest pressure difference found between upper and lower chambers is 0.5 mm. of water.

10. The conductivity method used (Newton, 1935) has been made sensitive to 0.001 mg. of carbon dioxide, the amount in 2 c.c. of ordinary air. Such air can be passed through the cell at 2 l. per hr. for 9½ hours before the absorbent needs to be replaced.

The author wishes to thank Professor F. G. Gregory, who originally suggested the 'through' experiments, for his constant encouragement and most stimulating criticism. Thanks are also due to Mr. Yardley of the Engineering Department, Chemical Research Laboratory, Teddington, for assistance in the detail design of the leaf chamber, to Dr. R. G. Newton for advice as to the conductivity method and to Mr. R. V. Martin of this Institute who did most of the glass-blowing and assisted in the erection of the apparatus. The main part of the work was carried out while the author was holding a Leverhulme Research Fellowship.

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EXPLANATION OF PLATE XIV

Illustrating Mr. O. V. S. Heath's paper on 'Experimental Studies of the Relation between Carbon Assimilation and Stomatal Movement. I. Apparatus and Technique'.

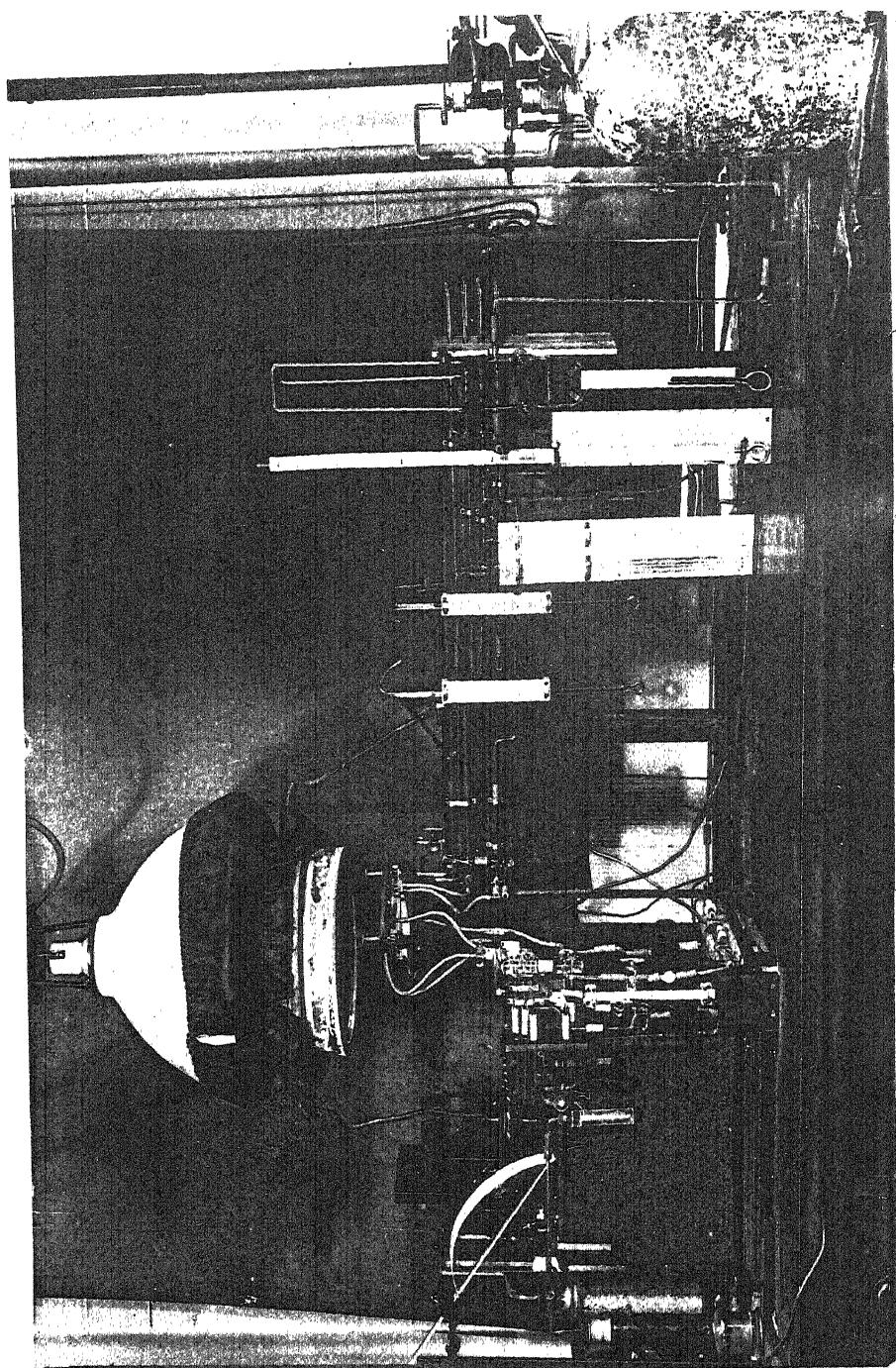
Fig. 1. General view of the apparatus used for the study of carbon assimilation and stomatal movement. The conductivity apparatus used for the determination of carbon dioxide in the air from the leaf chambers is not shown. (For details see diagrams and text.) (Photograph by H. Tooley.)

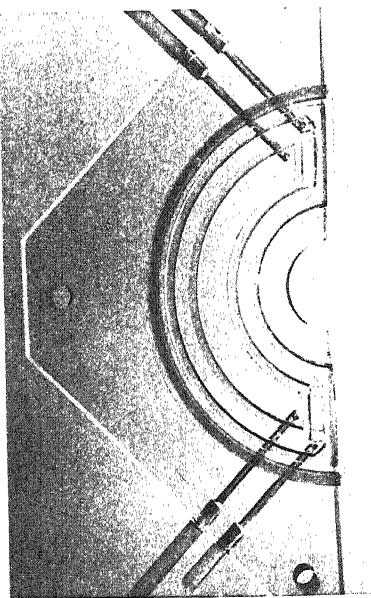
Fig. 2. General view of the leaf chambers, with gelatine washers and knife in position, showing method of support. Also brass mould for casting washers for the upper chambers.

Fig. 3. Brass mould for casting washers for the upper chambers.

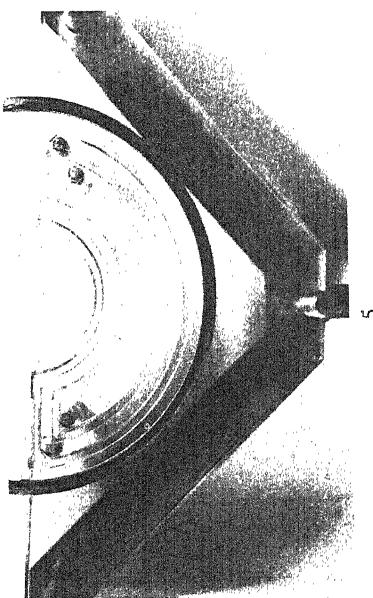
Fig. 4. Upper chambers, with gelatine washers in position.

Fig. 5. Lower chambers, with gelatine washers and knife for trimming leaf for 'through' experiments; the brass frame is shown.

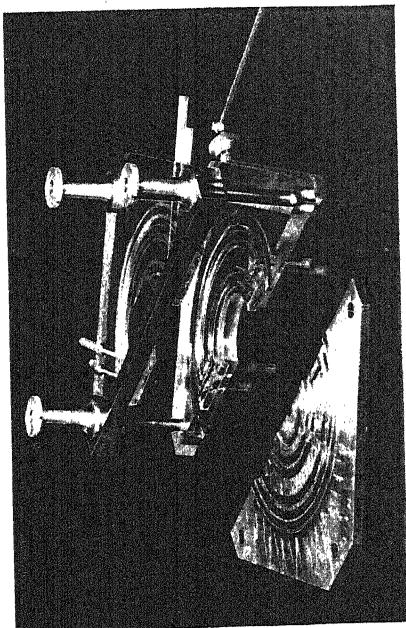




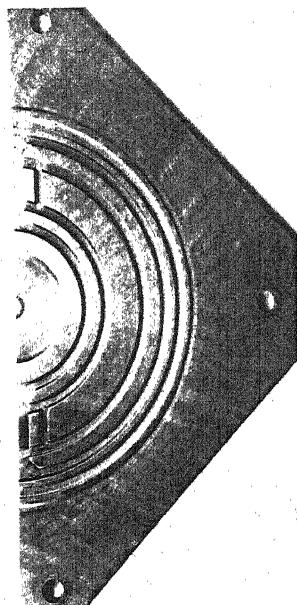
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HEATH — ASSIMILATION AND STOMATAL APERTURE.

NOTES

THE STAINING OF HERBARIUM MATERIAL OF CERTAIN SPECIES OF SELAGINELLA

INTRODUCTION

Herbarium material of several homophyllous species of Selaginella was obtained from the National Museum and Herbarium at Washington, D.C. Short lengths of cones, aerial stems, and rhizomes were 'resuscitated' by the method described by R. C. McLean (New Phyt., xv, 1916) but subsequently the staining of microtome sections of the material presented great difficulty. McLean mentioned similar difficulty and suggested acid fuchsin and light green in clove oil as a possible combination, but did not carry out further investigations.

With aqueous safranin and light green in clove oil a muddy effect was obtained owing to the dull red-purple colour produced in the xylem, and when aqueous Delafield's haematoxylin was used in place of light green there was little contrast between the colours shown by the xylem and the thin-walled cells. However, a solution of safranin consisting of equal parts of a 1 per cent. aqueous solution and a 1 per cent. solution in commercial absolute alcohol gave the normal brilliant red coloration in the older wood but left the protoxylem and foliar xylem almost unstained.

Methylene blue and erythrosin in clove oil also gave poor results, the former after a short staining period being removed by the clove oil and after a longer period remaining fixed in the cellulose walls.

A search was then made for suitable stains for the material to be investigated. Tests were carried out on microtome sections of the resuscitated materials with a large variety of stains and in the majority of cases mordanting was found to be essential. The cone tip of *Selaginella eremophila* Maxon, the aerial stem tip of *S. arenicola* Underwood, the cone tip of *S. asprella* Maxon, and the rhizome of *S. arenicola* were used. The last two were bleached before cutting by the method suggested by McLean. The bleaching process was extremely violent, causing a certain degree of maceration in the sclerenchymatous rind, and it was also impossible to ascertain when bleaching of the material was complete. If bleaching should be desirable it would seem preferable to employ a solution of chlorine for mounted microtome sections (Gwynne-Vaughan and Barnes, 'The Fungi', Cambridge Press, London, p. 381, 1937).

Details are given below of methods which proved satisfactory for the tissues under investigation.

GENERAL NOTES ON THE STAINING METHODS

Use of mordants.

Twenty minutes is given as the minimum time for mordanting and one hour may be regarded as the maximum. After treatment with a mordant the colours produced by the stains in the xylem were frequently rendered more brilliant as well as more

permanent than was otherwise the case, hence the desirability of using a mordant with certain stains even when no counterstain could be employed.

Washing in water.

If neither tap-water nor distilled water is designated it may be regarded as immaterial which is employed although running water indicates tap-water.

Staining.

No times are given for the staining periods and these must be found by experiment. Generally speaking, a long period is the more satisfactory, three hours being considered the normal maximum and fifteen minutes the minimum for the xylem stains.

The drying process.

Resort was only made to drying in air when the use of alcohol or a clove oil counterstain was prohibited owing to the rapid washing out of the xylem stain by these solvents even after mordanting. It has the disadvantage of tending to cause the disintegration of the phloem and endodermis in the young stems. After the drying process the slides should be kept in a slender jar of xylol for one hour or longer by which time the brilliant colour of the xylem is restored.

I A. *Iodine green (Grübler's Jodgrün). No counterstain.*

Treatment: Mordant with a 4% aqueous solution of iron alum or a } 20 minutes.
 Fresh 1% aqueous solution of stannic chloride
 Rinse quickly in distilled water.
 1% aqueous iodine green.
 Wash thoroughly in water. (Running water may be used.)
 Dry in air.
 Xylol. Canada balsam.

Result. This was a good method for protoxylem in young unbleached material. The xylem was here stained a blue-purple with microspores green and cellulose walls a pale violet. The more brilliant result was obtained following the use of stannic chloride.

When applied to relatively mature wood which had been bleached equally good results were obtained, but here all the xylem was stained a bright blue-green and the cellulose walls were colourless.

I B. *Iodine green. Erythrosin in clove oil.*

Treatment: 1% aqueous solution of iodine green.
 Rinse in water.
 Dehydrate rapidly with 80% alcohol and absolute alcohol.
 Erythrosin in clove oil.
 Xylol. Canada balsam.

Result. This method was only applicable to mature wood which was found in the bleached rhizome. The xylem was stained a dull blue-green with pink cellulose walls. When a 1 per cent. aqueous solution of stannic chloride was employed as a mordant before using iodine green this method was satisfactory for young unbleached xylem which was stained a deep blue-purple.

II. Cyanin (Baird and Tatlock). No counterstain.*Treatment:*

Mordants: A saturated aqueous solution of tannic acid,
or Fresh 1% aqueous solution of stannic chloride,
or 4% aqueous solution of iron alum.

Mordant for 20 minutes.

Rinse quickly in distilled water.

Fresh 1% solution of cyanin in 50% alcohol.

Wash thoroughly in water. Dry in air.

Xylol. Canada balsam.

Result. This method stained the unbleached protoxylem a bright blue after treatment with tannic acid or stannic chloride and a softer blue after iron alum. With the bleached material showing mature wood satisfactory results were obtained after the use of stannic chloride or tannic acid but not after iron alum. When stannic chloride was employed the wood was stained a brilliant blue with cellulose walls of a paler tone, while after tannic acid the wood showed a softer but still deep blue shade and the cellulose walls were colourless. Colourless cellulose walls would probably be obtained after the use of stannic chloride with a longer period of washing in water.

III. Basic fuchsin (Gurr's 'Special'). Light green in clove oil.*Treatment:* A saturated solution of basic fuchsin in commercial absolute alcohol.

Rinse in tap-water.

Dehydrate with 80% alcohol and absolute alcohol.

Light green in clove oil.

Xylol. Canada balsam.

Result. Satisfactory results were obtained with protoxylem and old wood which were stained a rich dark crimson. The green of the cellulose walls provided a good contrast.

IV A. Malachite green (Baird and Tatlock). Orange G in clove oil.*Treatment:*

Mordant: 8% aqueous solution of caustic potash. 20 minutes.

Rinse in distilled water.

3% aqueous solution of malachite green (at least two hours).

Wash in running water.

Dehydrate rapidly with 80% alcohol and absolute alcohol.

Strong orange G in clove oil.

Xylol. Canada balsam.

Result. A brilliant green stain was produced in the relatively mature unbleached wood but only a pale green coloration was present in the protoxylem. Cellulose walls were stained a golden yellow by the orange G. Rapid dehydration was essential for the success of this method as malachite green was readily washed out by alcohol and clove oil.

IV B. Malachite green. Orange G in clove oil.*Treatment:*

Mordant: 4% aqueous solution of iron alum. 20 minutes.

Rinse in distilled water.

Then as for method A above.

Result. This method gave the same colour results as did method A and was suitable for mature bleached wood, the xylem of the leaf traces being only lightly stained.

IV c. *Malachite green. No counterstain.*

Treatment:

Mordant: 4% aqueous solution of iron alum. 20 minutes.

Rinse in distilled water.

3% aqueous solution of malachite green.

Wash thoroughly in running water. Dry in air.

Xylol. Canada balsam.

Result. This method was only successful with mature bleached wood which was stained a brilliant green. Cellulose walls were colourless.

V A. *Anilin green (Grübler). Erythrosin in clove oil.*

Treatment: A saturated solution of anilin green (stain over night),

or { Fresh 1% solution of stannic chloride. 20 minutes. Rinse in distilled water.

Anilin green for shorter period.

Dehydrate rapidly with 80% alcohol and absolute alcohol.

Erythrosin in clove oil.

Xylol. Canada balsam.

Result. This method gave clear results in mature bleached wood which was stained a bright green in contrast to the pink cellulose walls. It was unsuitable for protoxylem of young material whether bleached or unbleached.

V B. *Anilin green. No counterstain.*

Treatment: Saturated aqueous solution of anilin green.

Wash thoroughly in water. Dry in air.

Xylol. Canada balsam.

Result. Protoxylem and metaxylem of young unbleached stems were stained a more uniform green than was obtained with malachite green, and this method was equally applicable to more mature wood. The method would be improved if a clove-oil counterstain could be employed since the thin-walled tissues remained colourless, but no success in this respect was obtained in the unbleached cone tip even after mordanting.

VI. *Safranin. Light green in clove oil.*

Treatment: A mixture of equal parts of a 1% aqueous and 1% solution of safranin in commercial absolute alcohol.

Wash off the excess of stain with 80% alcohol.

Dehydrate with absolute alcohol.

Light green in clove oil.

Xylol. Canada balsam.

Result. The safranin gave the normal brilliant scarlet stain in the mature wood of bleached and unbleached material. With regard to the protoxylem it was not a successful method on account of the light staining of this tissue.

VII. *Methylene blue (Baird and Tatlock). Eosin in clove oil.*

Treatment: 0.1-0.2% aqueous solution of methylene blue.

Wash quickly in 80% alcohol. Dehydrate with absolute alcohol.

Eosin in clove oil.

Xylol. Canada balsam.

Result. This method was only successful with mature bleached xylem which was stained a bright blue-green. Eosin in clove oil proved a brilliant counterstain, but the result was not permanent. Erythrosin should prove more satisfactory.

VIII A. *Methyl green (source unknown). Erythrosin in clove oil.*

Treatment:

Mordant: 4% aqueous solution of iron alum. 20 minutes.

Rinse in distilled water.

1% aqueous solution of methyl green acidified with a few drops of glacial acetic acid.

Dehydrate rapidly with 80% alcohol and absolute alcohol.

Erythrosin in clove oil.

Xylol. Canada balsam.

Result. This method was only applicable to more mature unbleached wood.

VIII B. *Methyl green. No counterstain.*

Treatment:

Mordant: Fresh 1% aqueous solution of stannic chloride. 20 minutes.

Rinse in distilled water.

1% aqueous solution of methyl green acidified with a few drops of glacial acetic acid.

Wash thoroughly in distilled water. Dry in air.

Xylol. Canada balsam.

Result. This was a good method for protoxylem and young metaxylem of young unbleached cones. The wood was stained a blue-purple and the cellulose walls a light purple. Equally good results were obtained with mature bleached wood which was stained a vivid blue-green, cellulose walls being colourless or light purple according to the degree of washing out in water.

VIII C. *Methyl green. Erythrosin in clove oil.*

Treatment: 1% aqueous solution of methyl green acidified with a few drops of glacial acetic acid.

Dehydrate rapidly with 80% alcohol and absolute alcohol.

Erythrosin in clove oil.

Xylol. Canada balsam.

Result. Satisfactory results were obtained for the bleached wood of the rhizome which was stained a deep dull purplish blue.

VIII D. *Methyl green. No counterstain.*

Treatment:

Mordant: 4% aqueous solution of iron alum. 20 minutes.

Rinse in distilled water.

1% aqueous solution of methyl green acidified with a few drops of glacial acetic acid.

Wash in running water. Dry in air.

Xylol. Canada balsam.

Result. This method was applied to young bleached material. Protoxylem was stained a deep violet, cellulose walls being colourless. Some colour would have remained with a shorter period of washing in water.

IX A. *Methyl violet B (Grübler). Erythrosin in clove oil.*

Treatment:

Mordant: 4% aqueous solution of iron alum. 20 minutes.

Rinse in distilled water.

1% aqueous solution of methyl violet.

Dehydrate rapidly in 80% alcohol and absolute alcohol.

Erythrosin in clove oil.

Xylol. Canada balsam.

Result. Satisfactory results were obtained when this method was applied both to immature unbleached xylem and to mature bleached wood. In the former case the protoxylem was stained a deep blue-purple, and in the bleached rhizome a bright violet colour was produced.

IX B. *Methyl violet. No counterstain.*

Treatment: 1% aqueous solution of methyl violet.

Wash in running water. Dry in air.

Xylol. Canada balsam.

Result. Good results were obtained by this method for protoxylem and more mature wood of bleached material. The staining was a deep violet with colourless cellulose walls.

X A. *Methyl blue (Baird and Tatlock). Erythrosin in clove oil or orange G in clove oil.*

Treatment: A saturated solution of methyl blue in commercial absolute alcohol.

Dehydrate rapidly with 80% alcohol and absolute alcohol.

Erythrosin or orange G in clove oil.

Xylol. Canada balsam.

Result. This method gave satisfactory results with mature bleached wood which stained a deep blue. Orange G as counterstain gave the better results on the whole.

X B. *Methyl blue. No counterstain.*

Treatment: A saturated solution of methyl blue in commercial absolute alcohol.

Rinse in water to remove excess of stain.

Treat with 20-40% alcohol until the blue colour is almost removed from the cellulose walls. (A higher grade of alcohol may be used if the washing out is very slow.)

Wash in water to stop the action of the alcohol.

Dry in air.

Xylol. Canada balsam.

Result. A deep blue coloration was obtained in the youngest wood of the bleached material, but a counterstain could not be employed. Methods IX A and B are of

interest since methyl blue is used as a counterstain for fixed material and does not wash out in alcohol.

E. M. DEBENHAM.

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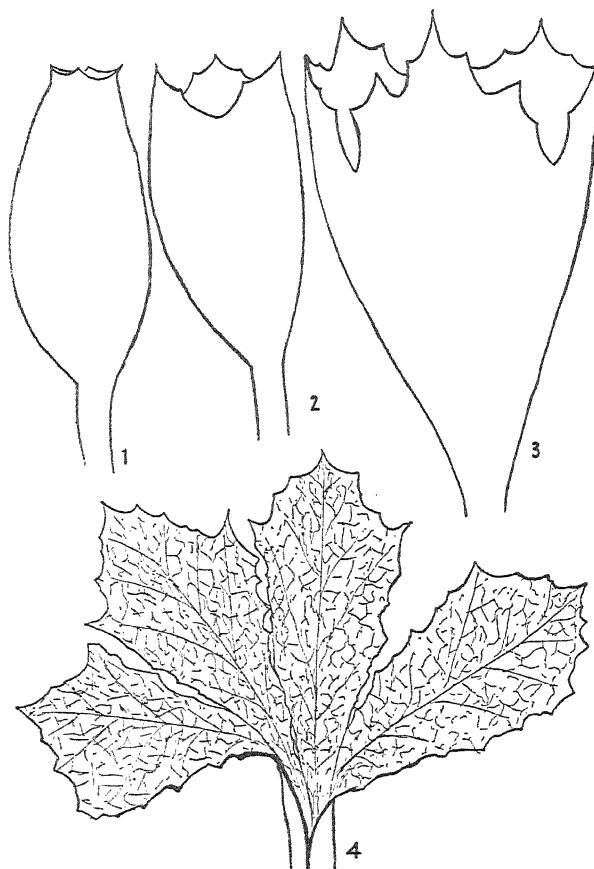
SOME ABNORMAL FLOWERS OF ARGEMONE MEXICANA AND THEIR BEARING ON THE MORPHOLOGY OF THE GYNOECIUM OF PAPAVERACEAE.—The morphology of the gynoecium of the Papaveraceae has been the subject of much controversy in recent years as has that of the related family Cruciferae. The generally accepted view that the carpels form one whorl of similar members and the placentae are joint outgrowths from their united margins has been attacked by Saunders (New Phytologist, xxix. 44, 1930) and Dickson (Journal of the Linnean Society, Bot. I. 175, 1935). From studies of vascular supply they have revived, in slightly different form, the old view of Kerner von Marilaun (The Natural History of Plants, Eng. trans., London, 1895), according to which the carpels in the Papaveraceae are of two types, an outer whorl of sterile carpels forming the wall of the ovary and an inner whorl of fertile carpels forming the placentae. Arber (Annals of Botany, N.S., ii. 649, 1938), on the other hand, from similar evidence supports the current view.

It might be possible to test these divergent views if by chance flowers could be found in some member of the family showing transformation of the gynoecium into leafy structures. The value of such teratological evidence in the solution of morphological problems has been a subject for much discussion among botanists. Its use, therefore, must be made with great discretion, but as Arber (Biological Review, xii. 157, 1937) says: 'It is an undeniable truth—indeed a truism—that aberrant forms, since they show what an organ can do, may sometimes throw light upon what it is.' Thus a syncarpous gynoecium completely modified into free leaves may not be of much value in deciding the nature of the carpels, but is certainly useful in deciding the number of the carpels which compose it.

Five years ago, the author (Joshi, Journal of Indian Botanical Society, xii. 255, 1933) described some flowers of *Argemone mexicana* L. from the Punjab showing partial phyllody. Both Saunders (loc. cit.) and Dickson (loc. cit.) postulate for this genus twice as many carpels as are generally accepted by systematists. A careful examination of that material, however, did not reveal any flowers in which the gynoecium had separated into its constituent carpels. No evidence therefore could be obtained concerning the composition of the gynoecium. Recently I have been fortunate in receiving material from South India, collected by Mr. J. Venkateswarlu of Andhra University in Cocanada during the summer of 1934, which helps in the solution of the problem.

The flowers in the South Indian material show all stages of phyllody. Those in an incipient stage agree with the flowers described previously (Joshi, loc. cit.) from the Punjab. They differ from the normal flowers in possessing persistent sepals, petals, and stamens. The petals are green to a varying degree. The stamens are further characterized by broader filaments and connectives, by the development of bristles on the filaments and of stomata and chlorenchymatous tissue in both, by the absence of the fibrous endothecium and of dehiscence of the anthers, and by abortive pollen. The gynoecium shows the development of a gynophore below the ovary, a

comparatively long style, freedom of the integuments of the ovules from each other and from the nucellus, a long micropyle, development of stomata on the integuments and of chloroplasts both in the cells of the integuments and of the outer layers of the nucellus, and the branching of the vascular trace of the ovules in the chalazal region.



Figs. 1-4. *Argemone mexicana*. Gynoecia from flowers showing phyllody. Figs. 1-3, three cup-shaped gynoecia as seen from outside. Fig. 4, a gynoecium completely transformed into free leaves spread out in one plane. $\times 5$.

In more advanced cases of phyllody the stamens are completely modified into leaves and the gynoecium begins to open out; the style and the stigmas disappear and the ovary forms a cup-shaped structure with a lobed margin (Figs. 1-3). The lobing in the early stages is only slight (Fig. 1); in flowers showing more advanced stages of phyllody this lobing is deeper and the lobes themselves are toothed (Figs. 2-3). In still more advanced cases, the gynoecium splits up into completely free leaf-like carpels (Fig. 4), and in these cases the ovules completely disappear.

The number of main lobes in these cup-shaped gynoecia is found to vary from

three to five. It is three in Fig. 2 and four in Figs. 1 and 3. The number of leafy carpels in gynoecia which had become completely foliar is also found to vary from three to five (Fig. 4). According to the theory generally adopted by the systematists the gynoecium of *Argemone mexicana* ordinarily consists of three to five united carpels. According to Saunders and Dickson, it consists of six to ten carpels. The evidence from the structure of the gynoecium of flowers showing complete phyllody thus supports the view generally held by systematists. It is against the views of Saunders and Dickson.

SUMMARY

Abnormal flowers of *Argemone mexicana* are described in which the gynoecium has been transformed into a cup with lobed margin or into free leafy carpels. The number of lobes or leafy carpels in such gynoecia is found to vary from three to five. This agrees with the general view of systematists regarding the number of carpels composing the gynoecium in this species, and is against the views of Saunders and Dickson who postulate twice as many carpels.

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A SIMPLE METHOD FOR OBTAINING PERMANENT DOUBLE-STAINED PREPARATIONS OF STARCH GRAINS AND THEIR ASSOCIATED PLASTIDS.—Four years ago Professor R. C. McLean (New Phytologist, xxxiii. 316, 1934) called attention to the xylol-soluble free-acid or free-base preparations of dyes such as eosin, methylene blue, &c., for use in anatomical staining. With the water-soluble and alcohol-soluble dyes now available reasonably good double-stained anatomical preparations can be obtained with so little practice that most botanists may not have felt the need of themselves employing or of instructing their students in the use of this alternative technique.

The present note is written to draw attention to the great value of one of these xylol-soluble dyes in a special case, that of obtaining permanent double-stained preparations of starch grains and their associated plastids. In the experience of the writer, the methods usually suggested are troublesome and, in the hands of students almost always unsuccessful. On the other hand, the method described below is simple, rapid and almost fool-proof, giving a sharp colour differentiation between plastid and starch grain, even more evident by artificial light than by daylight.

The method is as follows. Sections of the material, e.g., stem of *Pellonia Daveauana*, fixed in chrom-acetic or other suitable fixative, is stained for five to ten minutes in 0·1–0·2 per cent. methyl violet in water. Wash out excess stain in absolute alcohol until the starch grains are pale mauve. This takes five to ten minutes, so that differentiation is easily controlled. Transfer the sections to xylol to remove the alcohol and then place for a few minutes in xylol-soluble nile blue solution. (For method of preparation see McLean, loc. cit.) Rinse the sections in xylol to remove the surplus stain and mount in Canada balsam. The resulting preparation shows starch grains mauve and plastids gentian blue.

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Wall Structure and Growth

I. Spring Vessels in Some Ring-porous Dicotyledons

BY

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With Plates XV and XVI and eight Figures in the Text

INTRODUCTION

IN previous papers stress has been laid on the possible connexion between growth form in cells and the molecular structure of their walls. Such a connexion is clearly possible on two accounts. Study of the cell-wall is inevitably at the same time a study of cytoplasm, for the orientation of cell-wall material almost certainly reflects a corresponding orientation in the surface layers, at least, of the cytoplasm at some period in the history of the cell. It is, in fact, fairly clear in some cases (Preston and Astbury, 1937, and Chadefaud, 1933) that details of wall structures and of protoplasmic organization may correspond. From this point of view study of cell-wall problems are of importance, not for themselves alone, but because they represent to some extent a study of polarity in the cytoplasm. On the other hand, once the cell-wall is deposited, it forms a fairly rigid envelope surrounding each protoplast and separating the protoplasmic units completely, except for the so-called protoplasmic connexions (whose nature, however, is open to question: Jungers, 1930, 1933; Muhldorf, 1937). From this point onwards, the development of the cell is largely controlled by the cell-wall, either in its relation to internal and external mechanical factors or in its capacity for extension by growth alone. In other words, the shape and size of a cell are in some degree affected by the way in which the cell-wall itself can extend. The same considerations apply with equal force to tissues, and there can be no question but that studies of cell-wall structure are of considerable importance in phenomena occurring in tissue development. It is from this point of view that such studies are, in the last analysis, of any immediate fundamental importance.

The present investigation of the wall of the vessel was undertaken in the hope that the structure of the adult cell-wall would yield facts of importance in the elucidation of some of the outstanding problems in vessel development. Present conceptions concerning the differentiation of the vessel from the cambium are hardly so satisfactory as could be desired. In the comparatively uniform tissue of the cambium, individual cells suddenly undergo

comparatively enormous extension laterally to form future vessel elements. No adequate idea as to how this process is initiated, or as to the manner in which it is continued, has hitherto been presented. The generally accepted view that the causative factor is a sudden increase in turgor, which stretches the wall passively, is unsatisfactory in several regards, as will be pointed out below. Again, it is by no means certain how the very considerable accommodation of surrounding cells occurs, which must take place upon the sudden expansion of the future vessel element. The conception of sliding growth, so often invoked in such considerations, fails to present an adequate explanation. While the results to be presented are hardly such as to give a complete account of the principles underlying the phenomena involved, it is suggested that their interpretation does help to exclude some possibilities.

In carrying out an investigation such as this, it is necessary to survey the structure, not of isolated pieces of wall such as are seen in sections of material, but of the whole wall surface; and such a survey is clearly more valuable if the wall can be investigated as one whole area rather than dissected fragments. This is particularly true of the vessel element of which the wall has a sufficiently great extent to allow the possibility of variation in structure from point to point. A technique will be described which makes the necessary observations easy and accurate.

The component of the wall of chief importance from the present point of view is the polysaccharide cellulose. This is clearly the structural material of the wall around which the incrusting substances are deposited (lignin, pectin, hemicelluloses, &c.); for removal of these substances causes no fundamental change in such properties of the wall as its optical behaviour (Bailey and Kerr, 1935; Freudenberg and Dürr, 1932; Kanamaru, 1934), its cohesion (Bonner, 1936; Bailey and Kerr, 1935), and its molecular structure (Astbury, Preston, and Norman, 1935). The molecular structure has been worked out in considerable detail by the methods of organic chemistry and of X-ray analysis. It is unnecessary here to give any account of these results except in the briefest way. Cellulose consists of long chains of β -glucose residues bound together in the chain by primary valencies. The chains are aggregated by secondary valencies or van der Waals forces into bundles in which they are strictly parallel. These bundles correspond to the micelles of Nageli, though the present distinction between micelle and intermicellar space is not so sharply defined as in Nageli's classical view. It seems more reasonable to assume that the micelles represent vaguely defined regions in which the chains are parallel to each other, separated by regions of more or less random arrangement (Pierce, 1930; Astbury and Woods, 1932; Frey-Wyssling, 1935; and others). The cellulose chains in a cell are usually orientated with respect to some cell dimension. In ramie, for instance, they are approximately parallel to the length of the fibre; in cotton hairs (Balls, 1923; Frey, 1926), and tracheids (Frey, 1930; Preston, 1934), they may be inclined at a considerable angle; in vessels they are said to lie almost

transversely (Frey, 1930) and in some cells (e.g. *Valonia* (Preston and Astbury, 1937), *Cladophora* (Preston and Astbury, unpublished work), *Chaetomorpha* (Nicolai and Frey-Wyssling, 1938)) two directions of chains are clearly present. The generally accepted view that many cells of the xylem are included in this last category would, however, seem to be based upon rather scanty evidence. Such cases will be discussed briefly in the present paper.

The wall of the vessel is more complex than has generally been recognized. The work of Frey (1930; see also Jaccard and Frey, 1928) indicated a strictly transverse orientation of cellulose chains in the wall of the vessel element; yet peculiarities in the growth of such cells makes it rather doubtful if this is ever strictly true. It is found, in fact, that considerable variation may occur even in individual vessel elements; the wall structure is not uniform and certain of the modifications which occur are clearly correlated with growth processes. The work of Bailey and Vestal (1937), published during the preparation of the present paper, has already indicated some variability of this type in the structure of the walls of xylem elements; much of the variation which they observe is, however, undoubtedly due to the rather drastic treatment.

MATERIALS AND METHODS

Seven species were used in the present investigation. Specimens were obtained from the outer annual rings of the trunk wood of trees growing in the Arnot Forest, Ithaca, N.Y., U.S.A. The species used were as follows: *Quercus alba* L., *Qu. borealis* Mishx., *Fraxinus americana* L., *Castanea dentata* Borkh., *Celtis occidentalis* L., *Sassafras officinale* Nees. and Eberm., *Rhus typhina* L. The wood was macerated in 5 per cent. chromic acid at room temperature (75–80° F.), the vessels dissected out and mounted in glycerine. To facilitate extraction of the vessels, strips of wood some 10 mm. long and 1–2 mm. diameter were separated from the specimen and were threaded on glass capillaries or on fine copper wire by passing one of these through a vessel. After maceration the wire or capillary was then removed bearing a 'string' of vessel elements. It was thus a comparatively simple matter to fix the string of vessels to a glass slide, slit them longitudinally, and transfer them to a fresh slide in a drop of glycerine. Gentle manipulation of the cover-slip resulted in a rolling out of the vessel wall into a flat lamina. With care, about 20 per cent. of successes could be attained. This method has the double advantage of laying the whole wall-surface of the vessel element open to observation at the same time, and of making available the structure of the neighbouring vessel elements in a file. The slides were subsequently ringed with Canada balsam and have suffered no deterioration after more than two years. Observations were, however, always undertaken within a few days of preparation and were recorded as camera-lucida drawings of each vessel element.

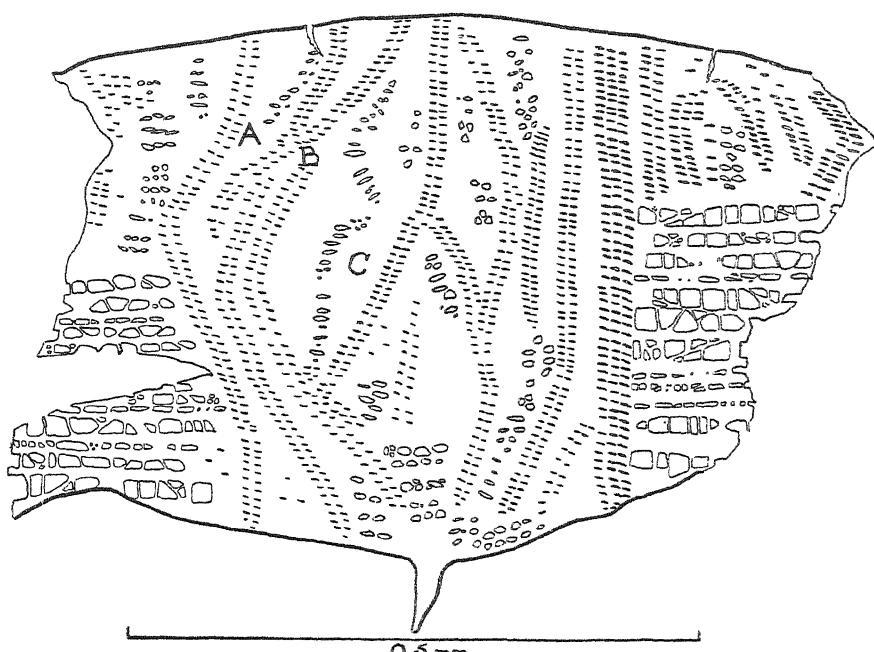
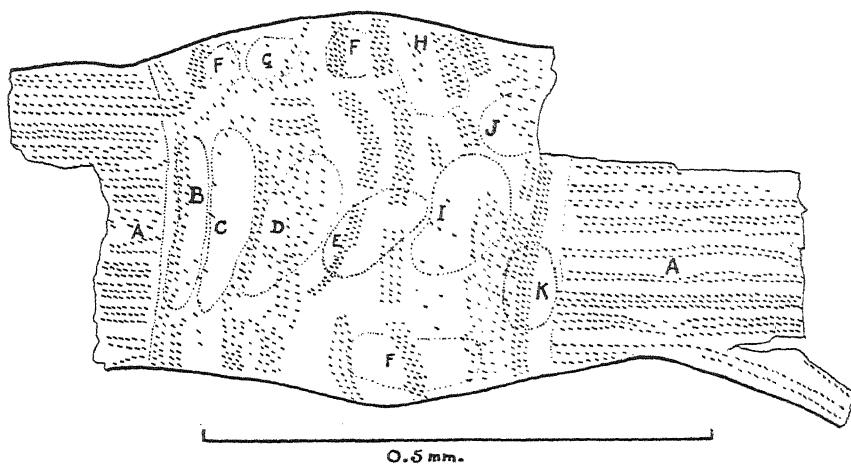
The observations deal with measurement of direction, and the first problem to be solved concerned the choice of a suitable axis of reference. Such an axis is preferably related to some definite cell dimension; in this case either the

length of the vessel or at right angles to it. As a first approximation, the direction of the files of ray parenchyma may be regarded as perpendicular to vessel length, a fact which may be checked by observation of the upper and lower edges of the vessel element seen in this view. The directions of the perpendicular obtained by these methods always checked within a few degrees, and the mean position was chosen. In the drawings which follow this axis is indicated by a solid line below each diagram. It should be emphasized that such details of pitting as are included serve to show only the distribution of the pits and their orientation; no attempt is made to introduce unnecessary features.

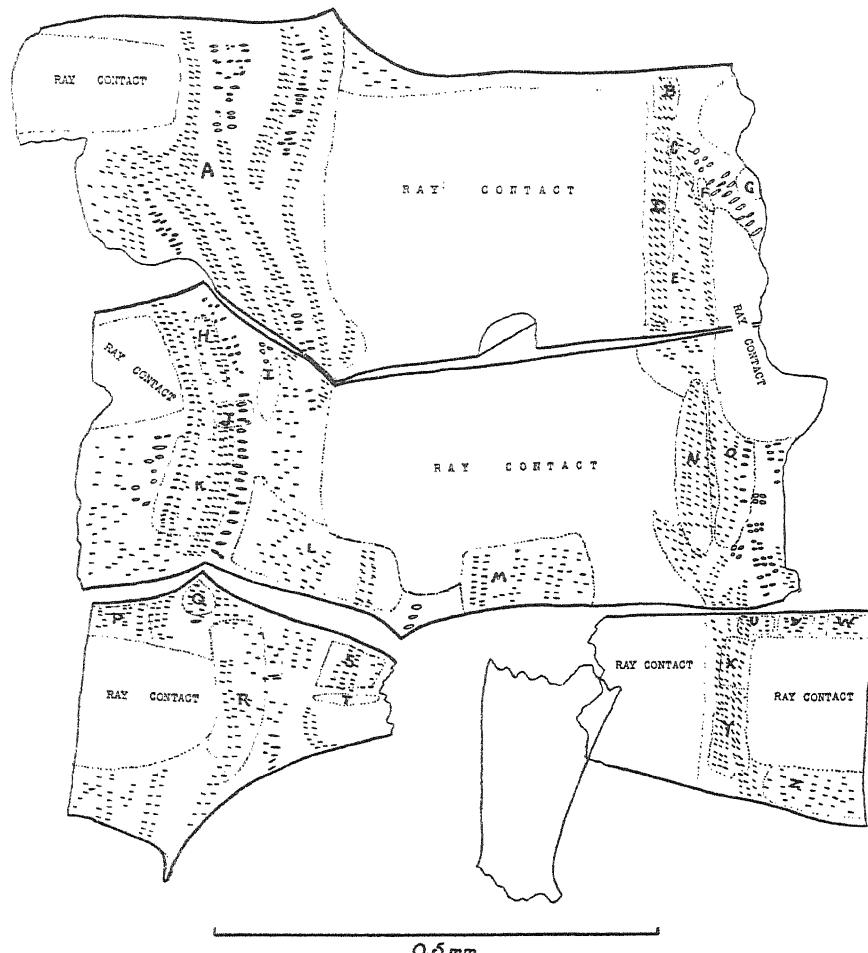
The bulk of the results to be presented refer to measurements made under the polarizing microscope. It is impracticable here to give any account of the principles involved—they may be obtained from any text-book on optics. The microscope used was a Leitz model and determinations were made with the aid of a subsidiary plate Blue II. In so far as it was necessary to use the methods of X-ray analysis, the microcamera was used to which reference has already been made (Preston, 1934).

THE DEVELOPMENT OF THE VESSEL

The most remarkable feature of vessel development is the enormous expansion which the vessel element undergoes, and considerable stress must be laid upon this point in any discussion of vessel-wall structure. For the moment it is of more immediate interest to note the effect of this expansion on the relation between the developing vessel and the neighbouring cells. The shape of the initial giving rise to a vessel allows the conclusion to be drawn that the maximum number of cells in direct contact with its longitudinal walls is twelve. In the expanded vessel, on the other hand, the number of contacts, as deduced from the pit arrangement in the figures to be presented, is considerably in excess of this. Disregarding ray contacts, it may be observed that in *Quercus borealis*, for example, as many as thirty-three contacts to tracheids and parenchyma are visible in a single vessel element (Text-fig. 1). If ray contacts are disregarded, the figures for the other genera are frequently lower than this (in Sassafras no measure can be given on account of the comparative lack of pits), but always in excess of twelve. An explanation of the increased number of contacts has been given by many workers in terms of 'sliding growth', whereby surrounding cells become elongated and move past one another. Certainly this elongation does play a part; vessels of *Quercus borealis* (Text-fig. 1), *Fraxinus americana* (Text-fig. 2), *Castanea dentata* and *Quercus alba* (Text-fig. 3) all exhibit tracheide or parenchyma contacts which, as judged by the grouping of the pits, are longer than the vessel element itself and occasionally longer than two contiguous elements. This would suggest, particularly in the case of the first-named species where the number of such contacts is so high, that vessel expansion is, in fact, connected with the elongation of surrounding cells.

TEXT-FIG. 1. Vessel of *Quercus borealis* (E₁).TEXT-FIG. 2. Vessel element (A₆) of *Fraxinus americana*. The dotted lines mark out areas in which the direction of the slit pits and the major extinction position are uniform (Table IV). No. 'spiral' pits.

That other processes are involved is also clearly indicated. It was early suggested (Krabbe, 1886) that cells adjacent to the expanding vessel are displaced laterally; and such displacement is certainly suggested by observations



TEXT-FIG. 3. Three contiguous vessel elements (upper B₁₇, central B₁₆, lower B₁₅) from the same vessel of *Quercus alba*. The dotted lines mark out areas as in Text-fig. 2 (Table IV). The pitting on the wall areas in contact with ray parenchyma is not shown.

on the present material. Generally we find two tracheid or fibre contacts meeting at the tips but otherwise separated by parenchyma (Text-fig. 1). Their appearance suggests that the two tracheids, originally in contact, are pushed apart and a cell previously farther out comes into contact with the expanding vessel element. This is a point of some importance, for if this interpretation is correct, then we have here a clear case of the secondary

formation of pits in a young expanding wall. The development of a new contact may obviously have occurred in either of two ways. The wall may have expanded, not uniformly but in localized areas—the sum total of the expansion causing an apparently uniform gross expansion—and the surrounding cells have been pulled apart; or the vessel wall may have expanded uniformly and glided over the surrounding cells which become distorted. In the latter case the majority of the pits are secondary in origin, while even in the former case the pits of new contacts are formed *de novo*.

One further point which calls for attention is the relatively enormous development of the ray contact, particularly in *Quercus* and *Fraxinus*, which the present method of observation brings into prominence. While the fusiform initial in the cambium could have made contact with only one ray initial at most, in the expanded vessel element we have frequently contacts to two rays, one on each side of the vessel element. Contact with the next ray along the periphery of the cambial cylinder is clearly made by a displacement of intervening initials during the original tangential expansion.

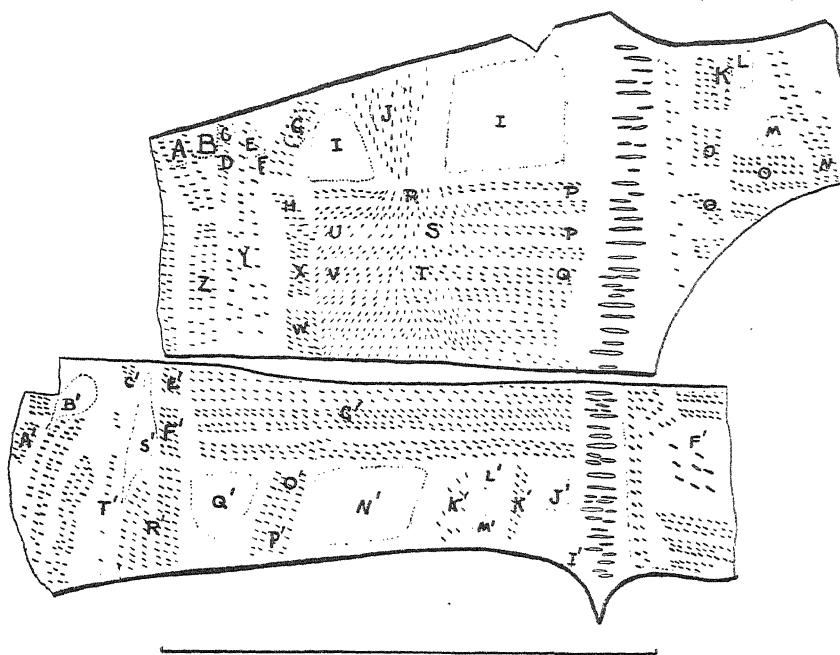
WALL LAYERING

In interpreting the observations which form the bulk of the present paper, certain points are to be borne in mind. The method of investigation involves the use of polarization phenomena, and the structure of the wall is determined from a study of its extinction positions. This type of observation must be regarded with suspicion unless supported by other evidence, for the major extinction position corresponds to the cellulose chain direction only if the wall consists of one set of such chains. The presence of more than one set can be detected by several methods, of which we may mention X-ray analysis, observation of the degree of extinction under the polarizing microscope, and optical analysis of cross-sections of the wall. The use of the first method will be noted in due course. In the second, use may be made of the fact that the degree of extinction is never so perfect with several wall layers, differing widely in chain direction, as with walls of only one layer (except when the layers are very thin and numerous (Preston, 1931; Astbury *et al.*, 1932; Preston and Astbury, 1937)); but it is difficult to judge degrees of extinction without the use of a photometer. With the vessels to be described below, the extinction is as perfect as could be expected from biological material, except in certain cases specifically mentioned. In the third method, the presence of layers in the wall with different optical properties is often used as a guarantee that more than one set of chains is present (Freudenberg and Dürr, 1932; Bailey and Kerr, 1935). It is, however, only safe to use this criterion if it is supported by evidence from other sources. Subsidiary methods such as the determination of the directions of striations and observation of the configuration of the canal in slit pits, while not of general utility, have been made use of in specific instances in the present considerations.

The walls of the majority of the spring vessels of the species studied here,

with the exception of Sassafras, are clearly composed of cellulose chains running in one direction only. The directions of the slit mouths of the pits are exactly coincident with the major extinction position in neighbouring areas of wall surface, and this makes it evident that any layer present with a chain direction different from that of the bulk of the wall thickness is negligible. The further demonstration that the wall is perfectly uniform in cross-section between crossed nicols (Pl. XV, Figs. 1-3) shows that such layers are, in fact, absent. This is again supported by the X-ray diagram of fragments of wall comparatively free from pits; only one set of diffraction spots is visible, corresponding to a single set of cellulose chains running approximately transverse (Pl. XV, Fig. 7). Unquestionably, the bulk of the spring vessels studied fall in this category. On the other hand, the walls of the remainder are certainly much more complex in nature. In Sassafras, for instance, nearly all the vessel walls fail to extinguish completely between crossed nicols, transmitting appreciable amounts of light at all azimuths; and many of them show other signs of layering in the present sense. In a few vessels the wall loses some layers during preparation. The thinner areas thus developed have extinction positions different from that of the remaining intact wall. This calls to mind a similar phenomenon presented by Valonia (Preston and Astbury, 1937) and allows a similar interpretation. These vessels in particular, and the majority of the vessels of Sassafras in general, must possess more than one direction of cellulose chains. This is supported by the fact that the vessel walls of this species are highly complex when viewed in transverse section in plane-polarized light (Pl. XVI, Fig. 13). On somewhat different grounds, a few vessel elements of *Fraxinus* may be shown to have a somewhat similar configuration. In clear areas, removed from pitted regions, the extinction is decidedly imperfect; while the pits themselves are of what we may term the 'spiral' type for the direction of the slits, as seen in surface view of the wall, varies from layer to layer (Pl. XVI, Figs. 11 and 12). This latter observation is of considerable importance from the present point of view. In those vessels whose walls are built up of a single set of cellulose chains the slits of the pits lie parallel to the chains. The presence of pits whose slit mouths vary in direction carries with it the necessary corollary that the wall is composed of more than one set of chains. Now careful examination of such pits in *Fraxinus* shows that there are, at most, three directions of the slit (so that its direction changes twice in focusing down through the wall) (Text-fig. 4; Table I). Here and there in some elements the layer nearest the lumen carries fine striations which are always parallel to the slits of the pits on this side of the wall. In all these cases the major extinction position lies, as one would expect, in the acute angle between the two limiting slit directions (Table I, here the angles marked as positive are those corresponding to which the cellulose chains run from bottom left to top right, when the wall is viewed from the inner face). In view of what follows it is of interest to note that the relative thicknesses of the layers may be estimated. It would be pointless here to attempt any precise computa-

tion, and it will suffice to point out that, in general, the major extinction position lies well to one side of the acute bisector of the angle between the (two) directions of the slit mouths. This means that in B_2 (Table I), the



TEXT-FIG. 4. Two contiguous vessel elements (upper B_3 , lower B_2) from a vessel of *Fraxinus americana*. The dotted lines mark out areas as in Text-fig. 2. The pits are universally of the 'spiral type', the direction of the slit mouths being shown only for the inner layer of the wall (see Table I).

central layer is generally the thicker, while in B_3 the inner layer tends to be relatively the thicker, with the exception of the extensive contact to ray parenchyma. In all cases studied, however, much variation occurs in individual vessel elements.

These apparently abrupt changes in chain direction may also be connected with the optical behaviour of a few of the vessel walls as seen in cross-section. While, as has already been pointed out, the majority of the walls are uniformly bright under the polarizing microscope, the existence here and there in *Fraxinus* of individual vessels, or small groups of vessels, whose walls obviously vary in brightness, or even in colour, is very striking. Such variations are, of course, merely visual manifestations of a change in *path difference*, which may be defined as

$$(n_\gamma - n_\alpha)d,$$

where n_γ and n_α are the greatest and least refractive indices, respectively in the wall section and d is the thickness. Characteristically, the wall is composed

TABLE I

Variation of the Direction of the Slit Mouths of Pits in *Fraxinus americana*

Vessel.*	Position.	Adjacent cell.	Inclination of the slit mouth to the transverse plane.			$\theta_i - \theta_o; \theta_o - \theta_c$	Inclination of the major extinction position.	Correlation coefficient (θ_i and θ_o both taken as +ve)
			Inner layer (θ_i)	Central layer (θ_o)	Outer layer (θ_c)			
B ₂ †	A'	w.p.	-16	+5	..	21	-4	-0.55
	B'	"	-4	
	C'	"	-35	+3	..	38	-9	
	D'	"	-32	-11	..	21	-22	
	E'	"	-45	-10	..	35	-22	
	F'	V.	-34	-5	..	29	-14	
	G'	r.p.	-38	-9	..	29	-22	
	H'	"	-19	
	I'	"	-4	
	J'	"	-48	-2	..	46	-5	
	K'	"	+6	
	L'	"	-5	
	M'	"	-21	
	N'	"	-25	
	O'	"	-57	-11	..	46	-25	
B ₃ †	P'	"	-38	-11	..	27	-25	-0.30
	Q'	"	-12	
	R'	"	-29	-4	..	25	-24	
	S'	"	-32	
	T'	"	-33	-4	..	29	-19	
						Average	32	
A	w.p.	-2	0	2	-2	-0.70
	"	-3	
	B	"	-10	-10	..	0	-9	
	C	"	-10	+17	..	27	+1	
	D	"	-10	+9	..	19	-3	
	E	"	-13	+10	..	23	-6	
	F	"	-34	+8	..	42	-17	
	G	"	-1	+7	..	8	..	
	H	"	-27	
	I	"	90	-12	..	78	-11	
	J	"	-37	0	..	37	-21	
	K	"	-7	
	L	"	-24	
	M	"	-10	
	N	"	-34	0	..	34	-15	
	O	"	-21	+2	..	23	+13	
	P	"	+13	+13	..	0	0	
	Q	"	-24	+20	..	44	-10	
	R	"	90	-4	..	86	-4	
	S	"	-84	+4	..	88	-4	
	T	"	-70	+10	..	80	-12	
	U	"	-20	-1	..	21	-2	
	V	"	+45	-13	..	58	+10	
	W	w.p.	-30	+11	..	41	-5	
B ₇	X	"	-10	+16	..	26	-2	-0.70
	Y	"	-6	+22	..	28	1	
	Z	"	-3	+23	..	26	5	
						Average	39.5	
	A	w.p.	+9	-14	..	23	-13	
	B	"	+9	-17	..	26	-10	
	C	"	+4	-22	..	26	-13	
	D	V?	+10	-13	..	23	-12	
	E	w.p.	..	+20	..	30	-6	
	F	"	+32	-14	..	46	-10	
	G	V?	+45	+5	..	40	+9	
	H	w.p.	+77	0	..	77	+14	
	I	"	+34	-7	+34	41	+10	
	J	"	+20	+2	..	18	+13	
	K	"	+26	-10	+20	36.30	+11	
	L	"	+18	-4	..	22	+7	
	M	r.p.	+40	0	+22	40.22	+5	
	N	"	+36	-3	..	38	+2	
	O	"	+15	0	..	15	+1	
						Average	30.0	

w.p. = wood parenchyma; r.p. = ray parenchyma; V = vessel.

* In this and other tables, the capital letter denoting a vessel element refers to one particular vessel. The subscript refers to the position of the particular element in the vessel, numbered from the first element examined.

† See Text-fig. 4.

TABLE II
The Double Refraction of Vessel Walls in 10 μ Transverse Sections

Element.	Adjacent cell type.	Path difference. (m μ).	Thickness of wall (μ).	n_r' .	Inclination of micelles.		
					Calculated.	Observed Element.	Angle.
<i>Fraxinus americana</i>							
Vessel 1	Vessel 2	A 193 ± 4 B 447 ± 8 C 202 ± 5	1.97 6.30 2.04	1.549 1.575 1.550	51° 16° 49°
Vessel 2	Vessel 1	A 267 ± 30 B 447 ± 8 C 202 ± 5	1.76 7.10 1.90	1.557 1.575 1.550	41° 16° 49°
Vessel 5	Vessel 6	A 333 ± 26 B 500 ± 33 C 191 ± 10	1.563 1.580 1.549	35° 0° 51°	B ₂ (Table I) ..	34° 5°
Vessel 6	Vessel 5	A 333 ± 24 B 500 ± 25 C 191 ± 12	1.563 1.580 1.549	35° 0° 51°
Vessel 7	Vessel 8	A 250 ± 19 B 500 ± 20 C 260 ± 33	1.12 4.30 1.38	1.555 1.580 1.556	45° 0° 44°	B ₇ (Table I)	A 45° B 5°
Vessel 8	Vessel 7	A 250 ± 19 B 435 ± 19 C 250 ± 19	3.75 4.45 1.12	1.555 1.573 1.555	45° 9° 45°		
Vessel 1	Ray parenchyma	A 126 ± 5 B 0 C 144 ± 6	0.78 v. thin 0.78	1.543 1.530 1.542	58° 90° 58°	See Text-fig. 4
Vessel 3	Ray parenchyma	A 366 ± 16 B 500 ± 10 C 346 ± 7	2.00 2.00 1.70	1.567 1.580 1.565	31° 0° 33°	B ₇ (Table I) ..	46° 36' 3° 22°
Vessel 3	Wood parenchyma	A 339 ± 16 B 500 ± 39 C 357 ± 7	2.22 3.58 2.73	1.564 1.580 1.566	34° 0° 32°	B ₇ (Table I) ..	34° 7° 34°
Vessel 1	Wood parenchyma	A 272 ± 6 B 447 ± 20 C 228 ± 6	0.52 3.20 0.84	1.557 1.575 1.553	41° 16° 46°	D ₁ ..	49° 7° ..
Vessel 8	Wood parenchyma	A 218 ± 18 B 0 C 218 ± 18	1.552 1.530 1.552	48° 90° 48°
<i>Castanea dentata</i>							
Vessel 1	Tracheid	A 247 ± 17 B 0 C 247 ± 17	1.36 0.68 1.36	1.555 1.530 1.555	45° 90° 45°
Vessel 1	Tracheid	A 346 ± 12 B 0 C 346 ± 18	1.36 0.70 1.70	1.565 1.530 1.565	33° 90° 33°
Vessel 2	Tracheid	A 334 ± 38 B 200 ± 15	2.7 ..	1.563 1.550	35° 49°
Vessel 4	Tracheid	A 116 ± 20 B 500 ± 48 C 233 ± 15	3.00 1.36 1.38	1.542 1.580 1.553	60° 0° 46°
Vessel 3	Ray parenchyma	A 366 ± 54 B 500 ± 60 C 233 ± 50	1.72 3.05 1.34	1.567 1.580 1.553	31° 0° 46°

A = inner layer.

B = central layer.

C = outer layer.

of three layers, although in a very few cases numerous fine lamellations can be detected which are so thin that 'halation' from neighbouring lamellae prevents the determination of the path difference. Wherever two vessels come into contact, the wall is always much thicker than elsewhere (Table II), and in those vessels which show the variations under consideration a large part of this increase in thickness is due to the development of a thick central layer,

whose path difference is much greater than those of either the inner or outer layers (Pl. XVI, Fig. 8). The same terms apply equally well to regions in contact with either wood or ray parenchyma (Pl. XVI, Fig. 9), when the wall is rather thicker than usual. On the other hand, the central layer of a wall in contact with elements other than a vessel is often rather thin and is to all intents and purposes isotropic (Pl. XVI, Fig. 10; Table II). Here, then, we have changes in wall structure both in surface view and in cross-section, which are clearly not unrelated. The only vessel elements observed to have spiral pits were found in a single vessel, and those showing variations in cross-section occur in strictly localized regions of the wood.

It seems reasonable to suppose that these latter variations are, in fact, due to changes in micelle direction. This assumption is supported by the agreement between the calculated changes in micelle direction, necessary to explain the variation in path difference, with the changes observed in face view of the wall—an agreement which is all the more striking since it is impossible to use the same vessel element for both types of measurement. The calculations may be based on the observation that, in face view, one direction of the slit pit is often approximately transverse, particularly in vessel to vessel contacts. It may therefore be assumed that the greatest path difference observed in section corresponds to a set of micelles running transversely, or sufficiently nearly so to reduce the error in subsequent calculations to a few degrees. In sections 10μ thick the maximum path difference observed (Table II) is 500μ , and the refractive indices n_α and n_γ of the micelles constituting the wall may be expressed as

$$(n_\gamma - n_\alpha)10 = 0.5.$$

It is unfortunately impossible to give a precise figure to either of these refractive indices individually, but a sufficiently close approximation may be made since, over a rather wide range of material n_α varies but little from the value 1.530 (Frey, 1926; Kanamaru, 1934). Hence to n_γ may be assigned the value 1.580, which seems a reasonable figure for lignified tissue (Kanamaru, 1934). If, now, the cellulose chains in other layers are tilted, rather than transverse, then the path difference will be

$$10(n'_\gamma - n_\alpha) = p, \text{ say},$$

where $p < 500\mu$, and the refractive index n'_γ may be calculated from

$$n'_\gamma = p/10 + 1.530.$$

The inclination of the cellulose chains to the transverse plane may then be calculated according to the equation

$$\sin \theta = \frac{n_\alpha}{n'_\gamma} \sqrt{\frac{n_\gamma^2 - n'^2_\gamma}{n_\gamma^2 - n_\alpha^2}}.$$

The angles thus calculated are presented in Table II, together with angles actually observed in Text-fig. 4 and Table I. All the angles calculated can be

matched against angles observed in similar regions of a vessel wall. On such grounds the conclusion is inevitable that the optical phenomena observed in sections are to be explained in terms of changes from layer to layer in chain direction.

The importance of such calculations lies in the fact that the conclusion thus attained may be generalized to cover some vessels in other species whose cross-sections possess identical features, although in no case other than *Fraxinus* has there been observed any signs of spiral pits or of crossed striae. Here again, however, the wall at a vessel to vessel contact is thickened as a consequence of the presence of a thick, central layer with a high path difference, while the wall at other contacts is generally thin with a central thin layer of low path difference. In all species any layer may differ in optical behaviour from point to point (Pl. XV, Fig. 5). This agrees with the observed changes in micellar direction.

Finally, it is a further significant and interesting feature of the walls of these less common elements that the directions of the chains in the layers of the wall (as measured by the direction of the slit pits) are not independent. There is a definite correlation in that the angle between them tends to remain constant (Table I). The average angle between the chains for each vessel investigated is, in addition, approximately the same, in spite of the comparatively wide variation in individual angles. If this is not a mere coincidence, it constitutes a fact of the highest importance. Similar results have been obtained from *Cladophora* (Preston and Astbury, 1939), though both here and in *Valonia* the average angle is 83° —significantly different from the present figure, though included in the range of variation. In both cases, however, it seems reasonable to suppose that a marked tendency exists for chains in neighbouring wall layers to make a constant angle to each other, particularly since alternate layers have essentially the same chain direction.

THE ORIENTATION OF CELLULOSE CHAINS IN THE WALL

We may, then, assume that the majority of the secondary walls of the spring vessels of *Quercus*, *Castanea*, *Rhus*, and *Celtis* are largely homogeneous with regard to cellulose chain direction, while a considerable proportion of those in *Fraxinus* and almost all in *Sassafras* have two or more layers differing in this respect. In the following pages reference is to be made primarily to those vessel elements whose walls show no evidence of this latter structural complexity, and on these grounds the observations are generally discussed in terms of cellulose chain direction rather than of the major extinction positions.

It may be said immediately that uniformly transverse orientation of cellulose chains in the vessel wall is highly exceptional. Only in two cases out of some 200 examined (40 of which were surveyed in detail) could transverse orientation be demonstrated, and the deviation of the remainder is too great to be accounted for by erroneous choice of a base line (Table III). In many vessels of *Fraxinus* the chains are uniformly inclined, constituting a spiral which may

TABLE III

Species	Inclination of slit mouths of pits to the transverse plane (degrees).				Species.	Inclination of slit mouths of pits to the transverse plane (degrees).			
	Vessel element.	Max.	Min.			Vessel element.	Max.	Min.	
<i>Quercus alba</i>	A ₅	-28	-8		<i>Sassafras officinale</i>	B ₃	+13	0	
	B ₅	+23	+4			B ₄	0	0	
	B ₇	-29	0			D ₂	+16	-6	
	B ₈	-40	-7			D ₃	+16	-6	
	B ₁₅	-40	-3			C ₆	+30	0	
	B ₁₆	-33	-4			C ₁₄	+14	0	
	B ₁₇	-45	-12			C ₁₆	0	0	
	B ₁₈	+60	0			D ₁	+9	0	
	B ₁₉	-21	0			B ₄	+19	0	
	D ₂	-58	-3			B ₆	+20	+2	
	D ₇	-35	-7			A ₂	+16	0	
	D ₈	90	+10			B ₄	-12	0	
	A ₇	+46	0			C ₁	+42	-40	
	A ₈	+31	0			C ₇	+40	0	
	A ₁₁	ca. 0				C ₈	+12	-4	
	A ₁₂	+24	+2			C ₉	-33	0	
	A ₁₄	+50	0			A ₅	+17	0	
<i>Castanea dentata</i>	B ₁	+49	+4			B ₁	-14	0	
	B ₂	-60	0			B ₇	+22	-5	
	B ₃	-10	0			C ₁	-24	-8	
	B ₄	90	-4			D ₁	+13	+3	
	B ₆	+23	+5			D ₅	+24	+5	
	A ₁	+32	+2			E ₁	+13	-2	
	A ₃	+33	+17			E ₂	-6	-2	
	A ₄	+20	-13						
	A ₅	+36	+16						
	A ₆	-59	-19						
<i>Fraxinus americana</i>	A ₇	+22	+12						
	B ₂	-48	-4						
	B ₃	90	0						
	B ₇	+45	0						
	D ₁	+40	0						

be right- or left-handed (the sign varies even from element to element in the same vessel); but in general large areas of the wall have a constant cellulose chain direction which varies considerably from area to area, quite independently of the type of cell with which the various parts of the wall make contact (Text-figs, 1, 2, 3; Table IV). It is somewhat remarkable that areas lying between contacts to cells which have been pulled apart during vessel expansion are not significantly different in chain direction from areas covering the same cells where they have remained in contact. Smaller deviations, due to the presence of pits are, of course, universal and are particularly striking in the case of the large, slightly bordered pits on walls adjacent to ray-parenchyma cells in *Quercus* and *Castanea* (Text-fig. 1). Some of the most obvious variations in inclination occur in the neighbourhood of ray contacts, a steepening being noticeable particularly on one side of the contact (Pl. XV, Fig. 4). The area of wall showing the greatest inclination to the transverse plane in-

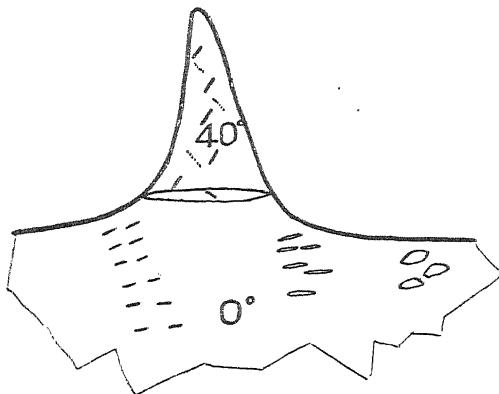
TABLE IV

Species. (Text-fig. 1)	Area (Text-figs.)	Inclination of major extinction position and/or slit mouths of pits, to transverse plane.		Species. (Text-fig. 3)	Area (Text-figs.)	Inclination of major extinction position and/or slit mouths of pits, to transverse plane.	
		Quercus <i>borealis</i>	Quercus <i>alba</i>			D	—12
<i>Fraxinus</i>	A	—8			B	—20	
	B	—2			C	—43	
	C	—4			D	—39	
	remainder	+13			E	—29	
					F	—17	
<i>Fraxinus</i>	A	—23			G	—45	
	B	—22			H	+4	
	C	—34			I	—10	
	D	—24			J	—4	
	E	—17			K	+4	
	F	—44			L	—10	
	G	—32			M	0	
	H	—59			N	—33	
	I	—39			O	—20	
	J	—19			P	+12	
	K	—34			Q	—34	
					R	—3	
					S	—4	
					T	+10	
					U	+10	
					V	+15	
					W	+12	
					X	—21	
					Y	—40	
					Z	—7	

variably lies between contacts to opposite rays rather than between two contacts to the same ray, and hence must correspond to what we may term the tangential walls of the vessel element; and of two areas in the wall lying between contacts to opposite rays, the narrower always has the steeper inclination. This sudden change in the direction of the micelles may be observed in any of the species studied, but is particularly noticeable in *Quercus alba*, where ray contacts are of considerable extent. This will be clear from Text-fig. 3, which also demonstrates the further point that the area of steepening lies always on the same side of neighbouring elements in a longitudinal file. In *Fraxinus* the same phenomenon occurs in the few vessel elements showing two ray contacts, whereas in *Quercus borealis*, *Rhus*, *Celtis*, and some elements of *Castanea*, no vessels of which have been observed with two ray contacts, a steepening may be observed on one side of the single contact. In *Sassafras*, on the other hand, where again two opposite ray contacts have not been detected, little signs of this steepening are to be observed. It is of particular interest that, where the phenomenon is most marked, the steepest chain direction in these areas adjacent to ray contacts is always nearer the contact itself, and often towards one end of the element. A similar steepening of the

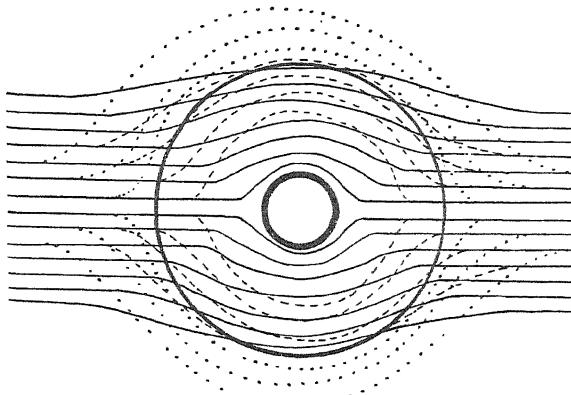
chain direction also occurs in the region of a 'tail' (Text-fig. 5) wherever such a structure is observed. This is continued in the 'tail' itself as a rather steep spiral.

The remaining variations in wall structure are apparently controlled by edges and may, perhaps, be termed 'edge' effects. They are of two kinds:

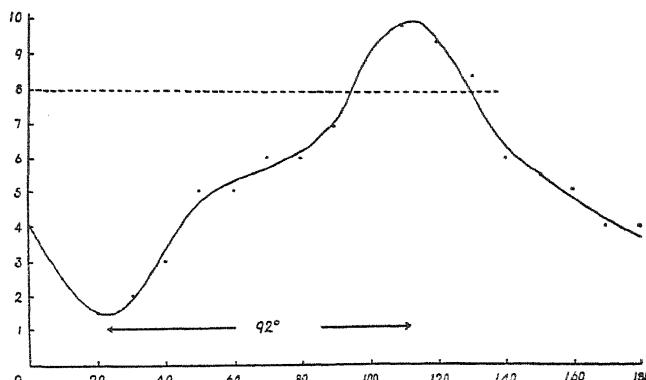


TEXT-FIG. 5. Vessel element of *Sassafras officinale*, showing the greater inclination of the slit mouths of the pits (and of the major extinction position) in a tail. The figures give the angle to the transverse plane.

those due to pitting and those brought about by the neighbourhood of the rim of the vessel perforation. The former is somewhat varied according to pit type. In the case of all pits with elongated mouths (as seen in surface view of the wall) (tracheid, vessel, and parenchyma contacts of *Quercus alba*, *Q. borealis*, *Fraxinus*, and *Castanea*, vessel contacts of *Celtis* and *Rhus*, parenchyma contacts of *Sassafras*) the chains run parallel to the edge along the side roughly parallel to the major axis of the pit mouth, and the extinction is somewhat sharper and the path difference greater here than anywhere else in the wall. At the 'points' of the pit mouth, although the major extinction position lies parallel to the edge, the extinction is by no means sharp, and the path difference is low. Occasionally, in fact, a small isotropic region may be detected at such points. The effect is more noticeable in fully bordered than in slightly bordered pits. Undoubtedly the effect is to be explained along the lines suggested by Scarth, Gibbs, and Spier (1929) and amplified by Bailey and Vestal (1937) except that in many cases the presence of more than one cellulose chain direction cannot be invoked. The effect here is clearly due to the encroaching of each consecutive lamella a little farther on the pit area than the last, the lamellae retaining the same chain direction (Text-fig. 7). A similar effect is readily observed with the circular bordered pits of tracheids in conifers, where the line joining the two positions of minimum path difference is parallel to the major extinction position of the rest of the wall. A curve relating the path difference to position in the border is presented in Text-fig. 6. It would seem that a similar curve may be taken to represent the position in the present



TEXT-FIG. 6. Hypothetical diagram of the run of cellulose chains in the neighbourhood of a bordered pit, in a wall built up of chains running in one direction only. Superimposed chains in three wall layers are considered. Dotted lines = chains in an outer wall layer. Broken lines = chains in an intermediate layer. Full lines = chains in an inner layer. The circles represent the limits of the border. The following points should be noted. On the upper and lower side of the diagram, the chains of the constituent layers run approximately parallel and the path difference will be correspondingly high. Towards the right and left, the chains cross one another at considerable angles and the path difference will be low. In intermediate positions the angle is smaller, and the path difference medium.

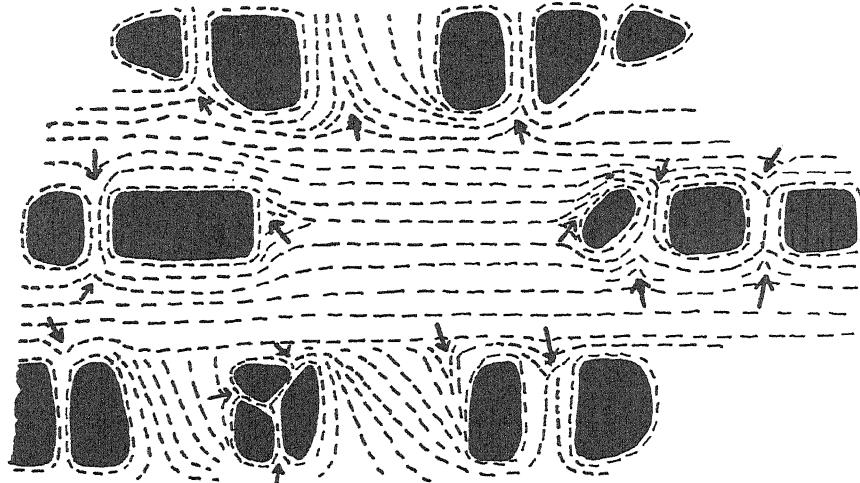


TEXT-FIG. 7. The change in birefringence with position of the border of a tracheid pit of *Abies nobilis*. Ordinate: readings of de Senarmont compensator. Abcissa: angular distance, in degrees, from the longitudinal axis of the cell. Compensator reading of neighbouring wall, 8 (broken line). Inclination of major extinction position to the vertical, in neighbouring wall areas, is 23° . This corresponds to the minimum of the curve.

type of pitting, though the eccentricity of the pit mouth and the interfering effects of neighbouring pits prevent accurate measurement.

Modification in wall structure due to the presence of pits may often be observed at considerable distances from the pit itself. In *Quercus alba*, for example, in a (pit-free) area lying either between two consecutive ray

parenchyma in a single radial file or between two pits in the same cell, the major extinction position depends to some extent on the distance apart of the first pits on each side. If these pits are far apart (Text-fig. 8) then the extinction position is roughly horizontal. As the pits approach each other, the extinction position becomes steeper (Text-fig. 8) until, when only a bar is left between them, it



TEXT-FIG. 8. Pits to ray parenchyma of a vessel element from *Quercus alba*. Black areas represent the pits, broken lines the directions of the major extinction position, arrows small, local areas of isotropy.

becomes vertical. Again, the effects of wood-parenchyma pitting is rather widespread. Wall areas in the region above and below the position of the parenchyma cell often show a major extinction position parallel to the edge of the nearest pit, while in those areas on each side of such a contact the position lies parallel to the direction of the long axis of the parenchyma cell itself, provided this is not too nearly vertical. This latter observation, however, is probably to be explained along other lines. A modification of this type of effect occasionally occurs, in which the wall around a group of pits has a configuration similar to that of the border in a single bordered pit.

Finally we have a peculiar effect of the rim of the vessel perforation. No matter what the chain direction in the rest of the wall may be, in the part of the wall nearest the rim (excluding the rim itself) it always changes so as to lie more nearly parallel to the rim at any particular place. This is readily observed in the bulk of the figures presented here. At the very edge of the wall, of course, the chain direction is always exactly parallel to the edge. Only one pronounced exception to this rule has been noted. With one vessel of Sassafras, the major extinction position in two places, one on the upper and one on the lower perforation and roughly on opposite sides of a diameter of the element, turns more or less abruptly to the vertical position. This is, however,

difficult to interpret on account of the almost universal complexity of the wall in this species.

DISCUSSION AND CONCLUSIONS

It is not possible at present to put forward any complete interpretation of the complex structure thus revealed. The question as to the existence of more than one direction of cellulose chains in the wall is obviously of extreme importance and perhaps demands some further attention. This is particularly true in view of the increasing prevalence of the view that plant cell-walls generally contain at least two layers differing in chain direction. Certainly this idea is fully justified in special cases (e.g. *Valonia*), but it is by no means safe to generalize from such results to cover cells of entirely different types. In some cases, the evidence for such a configuration is hardly convincing; this is particularly true of the wall structure of wood elements suggested by some authors. Suffice it here to point out that any interpretation based on swelling phenomena must be regarded with suspicion. Lateral swelling of an elongated cell in which the cellulose chains are arranged in a single spiral must cause a considerable flattening of the spiral, dependent on the degree of swelling; and if the wall contains lamellae which swell with difficulty, as seems probable (Bailey and Kerr, 1935; Ludtke, 1928, 1932), then the outer lamellae of a readily swollen layer will undergo a flattening greater than that of an inner layer. This, particularly if bursting occurs of the outer lamella in the development of 'balloons', will give the appearance of outer layers with approximately transversely, and inner layers with more or less longitudinally, orientated fibrils. This criticism would apply equally well to the work of Bailey and Vestal (1937).

Again, such considerations of the optical properties of the wall as have been used (Freudenberg and Dürr, 1922; Bailey and Kerr, 1935) are inadequate; for the observed phenomena may be accounted for either by a variation in chain direction from layer to layer, by a variation in chemical nature, by a change in the *dispersion* of a single direction, by a variation in the amount of cellulose present in the crystalline phase, &c. Any one of these possible causes may only be accepted when the others have been ruled out. Only a complete optical determination of the intact cell-wall will succeed in finally elucidating the precise nature of its structure. Preliminary results of such an investigation would indicate that variation in chain direction from layer to layer is, at least, by no means so extreme as has been supposed.

In the majority of the walls of the specialized vessels studied here, evidence of several different types has indicated the entire absence of a second set of chains. The extinction is as perfect as could be expected, the direction of the major extinction position is parallel to the direction of the slit mouth of the pit, the walls in cross-section appear uniform between crossed nicols, and in those elements of which an X-ray diagram has been obtained there are signs of only one set of diffraction images. The rarer cases in which cross-sections present the appearance either of bright and dark lamellae or of differently

coloured layers are certainly to be correlated with the observation of spiral pits (in *Fraxinus*) or of variation in extinction positions of walls denuded of successive layers (*Sassafras*). In the two genera mentioned, we are apparently justified in referring optical phenomena of cross-sections to a variation in direction of the cellulose chains constituting the wall.

The walls of the bulk of the vessels observed here are built up of rather flat spirals, instead of transverse cellulose chains as had previously been assumed. In spite of the wide variation which may exist in the inclination of this spiral on any one wall, this and other evidence clearly points to its derivation by flattening from an originally spirally wound initial. Thus it is found that the spiral in a tail is always much steeper than in the bulk of the vessel wall. Moreover, the micelles in the tangential walls of a vessel element are frequently more steeply inclined than elsewhere, and this steepening is often much more marked on one side of an element (generally the narrower of two areas lying between opposite ray contacts) than on the other. This would imply that the more steeply inclined wall is on the inner side which, on account of its close proximity to lignified elements, may be less expanded. Further support for this suggested flattening of an originally spirally wound cell is contained in the fact that in wood fibres, developed by considerable *elongation* from an essentially similar cell, the micelles are inclined very steeply. The facts are certainly suggestive, though at this date it is possible to put forward this suggestion only in the most tentative way.

Yet there are points in the observations which make it doubtful whether the *whole* of the vessel expansion is operative in flattening the spiral. Firstly, the whole expansion would be sufficient totally to flatten all but the steepest spirals. Secondly, continuous flattening under extension offers no explanation of the relatively wide variation of inclination in the same wall; and finally, the tangential walls, in the secondary layers of which the micelles are most steeply inclined, invariably expand to a considerable extent at an early stage. Some part of the extension must be such as to have little effect in flattening the spiral, and may even cause local steepening. It seems not unreasonable to suggest that a large part of the rapid early expansion of the vessel is due to growth of the wall rather than to extension by turgor forces; and that the growth processes involved are such as to have a local, rather than any marked general, effect. While growth in smaller cells may well cause changes similar to those caused by mechanical extension (Preston, 1938), modifications of a secondary nature will probably occur in cells of such large dimensions as those under consideration. The insertion of new wall-particles (or growth in the wall of particles already present) would lead to local disorientation rather than to ordered change in orientation. On these lines, the small areas showing rapid change in direction may obviously be correlated with areas of wall undergoing rapid growth, and in particular the steepening in the neighbourhood of ray- and wood-parenchyma contacts may well be due in part to the rapid growth of the wall in contact with these cells.

This conception of an early expansion by growth would also seem to remove some of the anomalies in the dynamics of vessel formation. If, for instance, the vessel is to be considered as developing under the influence of a mechanical force, then this force is clearly turgor pressure. It is often concluded that since the vessel is expanding and has liquid contents then it is fully turgid. This consideration is difficult to harmonize with the observation that developing vessels do not 'spray' when severed; and is not easy to reconcile with the facts of development. Of two adjacent cells in the cambium, certainly not very dissimilar as regards suction pressure, one suddenly begins to expand. This would necessitate a sudden increase in suction pressure (S). If the expansion of the cell is due to turgor, then we should also expect an increase in turgor pressure (W); and since

$$S = P - W,$$

this would necessitate an increase in P , the osmotic pressure of the cell-sap, i.e. a fairly rapid preferential absorption of solutes. Although it is clear that rapidly growing tissue can accumulate solutes to high concentrations, the hypothesis that one cell of the cambium can excel another in this regard would seem not to offer the simplest solution. If we postulate that a single cell suddenly begins to undergo expansion as a result of active growth of the wall, then this continued growth would necessitate a decreased W and an increased S . If W approaches zero, then $S = P$ initially, and S may then even decrease from this value so long as it does not fall below ($P - W$). There is, in other words, no need for the rapid absorption of solutes. We have merely to postulate an initial stimulus to growth, and though it would be unprofitable at present to speculate on the nature of the stimulus, it is significant that resumption of meristematic activity commences at a stem-growing point in the spring, and travels downwards in approximately vertical lines. On the whole, then, during this suggested early growth stage the inclination of the micelles in the wall must not be markedly decreased; any changes which do occur cannot be in proportion to the change in dimension which occurs. The flattening of the spiral must be attributed to a late expansion by mechanical forces. The steeper inclination in the tangential walls is then maintained by a failure of the vessel, situated between two adjacent rays, to expand as much tangentially as radially. The variation in the angle between cellulose chains in the various layers of some vessels of *Fraxinus* would, in fact, suggest that mechanical extension occurs even during the earlier stages of wall thickening.

The conceptions thus outlined concerning the relation of wall structure to growth processes carries with it the necessary assumption that the orientation of cellulose chains in the secondary wall is governed by that in the primary. The observations made in the present paper do, in fact, constitute a strong argument in favour of this view. On somewhat similar lines, the cambium of the conifer has been shown to possess a spiral structure analogous to that suggested here for the initials in the ring-porous dicotyledonous tree; and clear

evidence has been obtained that some primary walls are composed of cellulose chains arranged in a spiral (Preston, 1938) in spite of previous statements to the contrary (Bonner, 1936).

At the same time, some variations in wall structure certainly refer to the secondary wall alone. This is clearly true of the border of the pits as well as variations near to pits and obviously due to their presence. These are undoubtedly connected with the fact that the cytoplasm remains in contact with the wall in the region of the pit area much longer than with the rest of the wall, so that, figuratively speaking, the protoplasmic membrane here takes the form of a short, hollow cylinder, rather than a flat lamina. Similarly, the tendency of cellulose chains to lie parallel to a neighbouring region of the rim is possibly to be ascribed to the shape of the cytoplasmic surface, though it may equally well be explained in terms of the bursting and subsequent contraction of the cross wall of the developing element.

As regards those elements the direction of the cellulose chains in whose walls has been found to vary from layer to layer, the same conceptions may be invoked even though the mechanism by which the change in direction is produced is no more readily understood than with other types of cell showing the same phenomenon. On the basis of the correlation coefficients, it would seem probable that the direction in successive layers is not independent, even though the angle between them does actually vary over such wide range so that even here the primary wall may influence secondary layers to some extent. Nevertheless, it is difficult to see precisely how the observed structure is developed, for a considerable expansion of the element would seem to be necessary during secondary wall deposition.

SUMMARY

Examination, under the polarizing microscope, of vessel elements in some ring-porous dicotyledonous trees shows that the bulk of the elements is composed of cellulose chains lying in one direction only. These cellulose chains form a rather flat spiral and not a series of transverse circles. The inclination of the spiral varies widely in any one element, a steepening being very marked on some of the tangential walls. It is suggested that the structure of the secondary wall is best explained by a rapid, early extension, resulting from an active growth of the wall, of a spirally wound cambial initial. This early phase of development, which has little general effect on the inclination of the spiral, is followed by a period of extension by mechanical forces, leading to considerable flattening of the spiral. The variations in the inclination of the spiral are considered to arise during the early growth phase. These conceptions are further shown to be in harmony with the facts of vessel development.

In all species examined, and particularly with *Fraxinus* and *Sassafras*, the elements of a few isolated vessels possess walls which are more complex. At least three layers are visible in cross-section which, especially in *Fraxinus*, are to be explained by a change in micellar direction from layer to layer. Com-

monly, if the wall is thicker than usual then the micelles of the central layer are arranged almost transversely; if the wall is thin, they are arranged longitudinally. It is suggested that these unusual vessel types may also be explained by the course of development outlined above.

ACKNOWLEDGEMENTS

The author wishes to express his indebtedness to Professor J. H. Priestley, of the Botany Department, University of Leeds, for the benefit of helpful discussions during the preparation of the paper. His thanks are also due to Professor Guise, of the Forestry department, Cornell University, Ithaca, N.Y., U.S.A., for the specimens used, and to Professor W. C. Muenscher, of the Botany Department there, for his identification of the samples.

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EXPLANATION OF PLATES XV AND XVI

Illustrating Dr. R. D. Preston's paper on 'Wall Structure and Growth. I. Spring Vessels in some Ring-porous Dicotyledons.'

PLATE XV

Fig. 1. *Castanea dentata*. Transverse section of a vessel in plane polarized light (crossed nicols). ($\times 400$.) The vessel wall is perfectly uniform and shows no sign of layering.

Fig. 2. *Fraxinus americana*. ($\times 400$.) Conditions of illumination as in Fig. 1. The vessel wall in contact with ray parenchyma is uniform.

Fig. 3. *Fraxinus americana*. ($\times 400$.) Conditions of illumination as in Fig. 1. The wall in contact with a neighbouring vessel is uniform. The faint, dark lamellations are due to very slight changes in birefringence.

Fig. 4. *Quercus alba*. Photomicrograph of part of the wall of a vessel element showing the considerable increase in the inclination of the slit mouths of pits in the neighbourhood of a ray contact.

Fig. 5. *Castanea dentata*. Conditions as in Fig. 1. Note that the vessel wall shows distinct signs of layering and that a layer which is bright in the centre of the photograph becomes dark towards the lower right.

Fig. 6. *Castanea dentata*. Conditions as in Fig. 1. Although the intact wall is uniform in appearance, layers have been separated from it during sectioning. This occasionally gives an illusory effect of layering in the present sense.

Fig. 7. X-ray microphotograph (enlarged) of a single wall of a vessel element of *Quercus alba*. Only the diffraction spots from planes of $3\cdot9\text{A}$ are visible, but these suffice to show that there is but a single row of such equatorial spots and therefore only one set of cellulose chains in the wall.

PLATE XVI

Figs. 8–11 are transverse sections of vessels in polarized light ($\times 400$).

Fig. 8. *Fraxinus americana*. The wall in contact with another vessel has three layers. The illusory appearance of five layers (three bright and two dark) in each wall is due to a transition from yellow to blue via red in passing either from the inside or the outside of the cell towards the central thick layer.

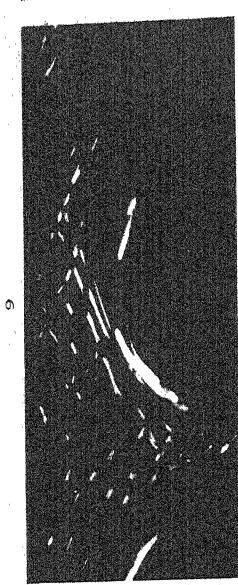
Fig. 9. *Fraxinus americana*. Three layers in the wall of a vessel in contact with wood and ray parenchyma. The central layer, which appears dark in the photograph, actually exhibited a colour Red I as against Yellow I of the layers on each side. The central layer, therefore, represents a region of higher birefringence.

Fig. 10. *Fraxinus americana*. The wall in contact with wood parenchyma here has a central, dark layer which is extremely thin. This is a true dark layer of zero path difference.

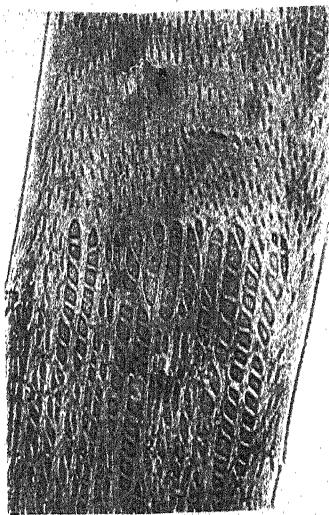
Fig. 11. *Fraxinus americana*. A group of pits, on the wall of a vessel where contact is made with a wood-parenchyma cell, as seen on a layer nearer the lumen.

Fig. 12. The same area of wall at a different focus, showing the appearance of the pits in a layer farther from the lumen. Note that the inclinations of the slit mouths are considerably different from those of the same pits in Fig. 11.

Fig. 13. *Sassafras officinale*. Note the complexity of the wall. Six layers are clearly visible on the right.



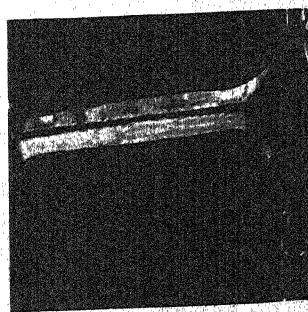
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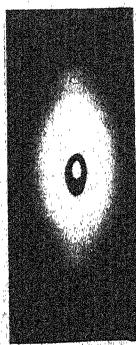
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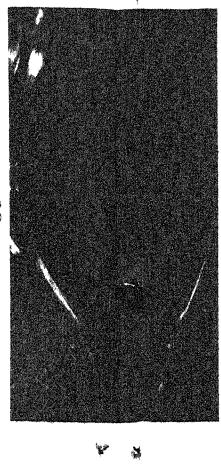
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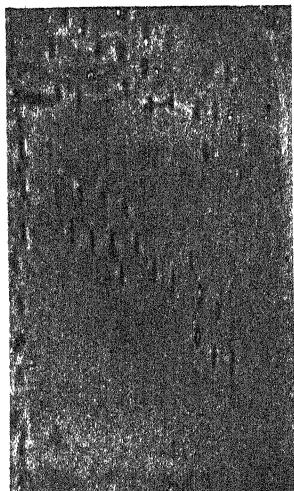
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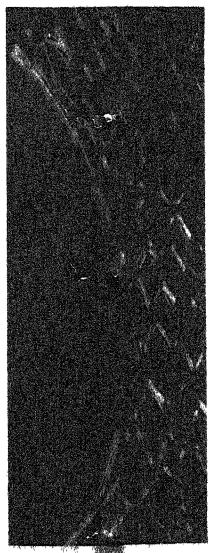
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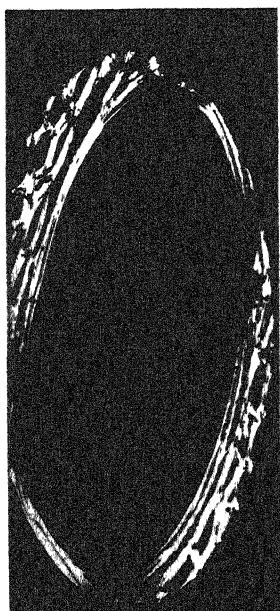
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9

Experiments on the Extraction of Sap from the Vacuole of the Leaf of the Cotton Plant and Their Bearing on the Osmotic Theory of Water Absorption by the Cell¹

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With Plate XVII and two Figures in the Text

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I. INTRODUCTION

IN a recent paper we (Phillis and Mason, 1937) suggested that the sap expressed from the leaves of the cotton plant by direct pressure in a hydraulic press is derived from the vacuole. It was found that even with dial readings of 16,000 lb. per sq. in. only about half of the total sap obtainable from leaf tissue previously frozen, boiled, toluened, or ground could be extracted from untreated leaves. Sap could readily be obtained from the residue at very low pressures after preliminary treatment by freezing, &c. It was also found that gently rubbing the residue between the fingers and thumb enabled sap to be expressed from it by hand pressure. There appeared to be two distinct types of sap. One, presumed to be vacuolar, is perfectly clear and colourless, while the other from the residue is always coloured; reddish-yellow from younger leaves and reddish-brown from older ones. It was suggested that the sap obtained from the residue is derived mainly from the dead protoplasm. The concentration of solutes in the vacuolar sap is much less than that in the residue sap. The pH value of the vacuolar sap usually lies between 7.0 and 7.2, while that of the residue sap may be as low as 5.7. It was observed that the concentrations of solutes in successive fractions of the vacuolar sap expressed from the living leaf in the hydraulic press

¹ Paper No. 18 from the Physiological Department of the Cotton Research Station, Trinidad.

remained constant and were independent of the pressures employed for expression.

To explain these observations, we suggested that the vacuolar sap escapes *unaltered* through fissures in the protoplasm when pressure is applied, and that the residue sap arises from the *decomposition* of the protoplasm and is of course diluted with any vacuolar sap not expressed. The continuous medium of protoplasm was conceived to be non-aqueous throughout (cf. Lepeschkin, 1930) and not only at the hypothetical surface membranes (cf. Osterhout, 1936). Protoplasm was further assumed to possess a relatively gross structure which is destroyed by small shearing forces but not by quite large direct pressures. Our observations seemed to accord better with the 'Vitaid' hypothesis of Lepeschkin (1936), which conceives protoplasm as a loose chemical combination of proteins and lipoids with which water is presumably in some way united,¹ than with the view that the continuous medium of protoplasm except at the surface membranes is aqueous. Protoplasm, as we (Mason and Phillis, 1937) have recently suggested, appears to possess the properties of a liquid with solubilities and diffusion constants quite different to those of water. On decomposition it appears to break up into its constituent parts, and those products that are soluble in water dissolve in the decomposition water to form the sap which we will refer to as the protoplasmic (or residue) sap.

The object of the present paper is to examine some of the implications of this interpretation of our observations on sap extraction and to consider further observations relating to the uptake of water by the residue after the vacuolar sap has been expressed. The term *residue* will be used to indicate that fraction of the leaf from which no further sap can be expressed by means of direct pressure in the hydraulic press.

II. THE DISTRIBUTION OF WATER BETWEEN THE VACUOLE AND THE PROTOPLASM

We have two methods of calculating the distribution of water between the vacuole and the protoplasm. The first estimate depends on the concentrations in the vacuolar sap, the protoplasmic sap, and in the sap extracted from the whole cell. The second estimate is obtained from extrapolation of the curve relating the pressures used in expression and the weights of water in the sap obtained for successive uniform increments of pressure.

To obtain the vacuolar sap, the large veins are removed from about 100 gm. of leaf material. The remainder of the lamina is then carefully arranged in a wad about 4 in. square and of uniform thickness, so that when pressure is applied there will be no tendency for the leaves to slip over one another, as this will introduce shearing forces which will lead to decomposition of the protoplasm and so release its water and solutes. It is more difficult to avoid

¹ A union which is possibly dependent on the expenditure of metabolic energy as well as on imbibitional forces.

shearing in old than in young leaves, and in those that are turgid than in those that are wilted. The wad of leaves is wrapped in cloth and placed in the hydraulic press. The pressure is then steadily applied. Usually two to three minutes elapse between the first application of pressure and the attainment of sufficient pressure to cause sap expression. The sap is collected in at least two fractions and should be colourless and clear. Where differences in concentration occur, disintegration of the protoplasm is judged to have taken place, and the lower concentration is assumed to be representative of the vacuole. Useful indicators of protoplasmic decomposition are the expression of coloured sap and the tearing of the wad.

It is not possible to express the whole of the vacuolar sap as the cloth usually bursts at dial readings below 16,000 lb. per sq. in. As the unexpressed vacuolar sap would dilute the sap released from the protoplasm, we wash out these remnants of the vacuolar sap by bringing about partial decomposition of the protoplasm by gently rubbing the residue between the fingers and the thumb and pressing out the mixed vacuolar-protoplasmic sap. The residue from finger rubbing and pressing is then frozen at -16° C. and the remainder of the sap released. This fraction is assumed to be derived from the decomposition of the protoplasm.

The concentrations of chlorine and of potassium in the vacuole and protoplasm respectively in two samples of leaf are shown in Table I. The concentrations in sap expressed from tissue exposed to preliminary freezing at -16° C. are also shown. This sap is assumed to represent the average concentration in the whole cell. From these three concentrations the amount of water which is resident in the vacuole has been calculated as a percentage of the total free water¹ in the whole cell.

TABLE I

Concentrations (mg. per 100 gm. water) of Chlorine and of Potassium in Vacuole, Protoplasm and Whole Cell; and Percentages of Total Water (sap) in Vacuole Calculated from these Concentrations.

	A	.	.	Vacuole.	Protoplasm.	Whole cell.	Percentage of total water (sap) in vacuole.
Chlorine	A	.	.	93	513	385	30.5
	B	.	.	103	493	369	31.8
	Mean	.	.	98	503	377	31.7
Potassium	A	.	.	135	768	590	28.1
	B	.	.	154	765	591	28.5
	Mean	.	.	145	767	591	28.3

¹ Water united with the vitaid molecule, and which is liberated on decomposition of the protoplasm, cannot in the living cell be present as free water, but is regarded as such in the present calculation. True bound (Mason and Phllis, 1936) or colloidal water does not enter into the above calculation.

It will be observed that the calculation indicates that only about 30 per cent. of the sap-water is present in the vacuole. In many text-books it is stated that the protoplasm occupies only a very small part of the mature cell, but measurements (cf. Iljin, 1934) do not support this view. In this connexion it is worth emphasizing that in a spherical cell, volume varies as the cube of the radius and that the thickness of the protoplasm need only be approximately one-fifth of the diameter of the vacuole for the volume of the protoplasm to be double that of the vacuole.

There is, however, as we have mentioned, another method of calculating the distribution of water between the vacuole and the protoplasm. One hundred grammes of leaf material similar to that used in obtaining the results given in Table I was prepared and placed in the hydraulic press in the way we have just described for the expression of vacuolar sap. The distribution of water, as determined on comparable samples of material, is shown in Table II. Bound water was determined by the chlorine method (Mason and Phillis, 1936). No sap was expressed until the pressure reached 6,000 lb. per sq. in.

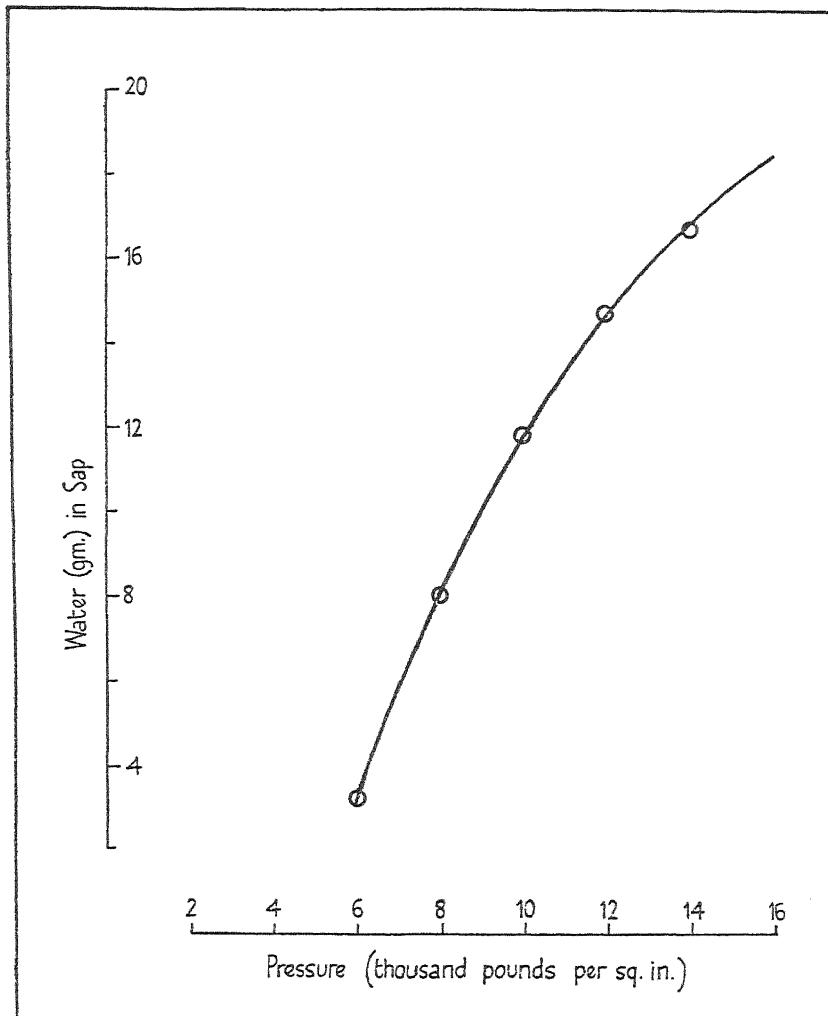
TABLE II
Distribution of Water (gm. per 100 gm. fresh weight)

Total	Bound.	Free.
76·4	4·8	71·6

The pressure was then increased by 2,000-lb. increments up to 14,000 lb., three minutes being allowed for sap-expression at each pressure. The total weights of sap-water expressed at each level are shown in Text-fig. 1. The increments for each 2,000-lb. increase were found to form a geometric series, and the curve shown in Text-fig. 1 is that calculated for such a series. It will be noted that the experimental points fit the curve very well. The maximum weight of water that could be expressed from the vacuole has been calculated from the equation $S_a = \frac{a}{1-r} + b$, where a represents the weight of water expressed between 6,000 lb. and 8,000 lb., r the ratio of the weights at each step of 2,000 lb., and b the weight expressed up to 6,000 lb. The theoretical maximum weight that could be expressed from the vacuole from the 100 gm. of tissue amounts to 24·2 gm. Actually 16·8 gm. (see Text-fig. 1), approximately 70 per cent. of the total calculated water content of the vacuole, was expressed. The *total* free water (see Table II) amounted to 71·6 gm. per 100 gm. fresh weight. Thus the percentage of the free water in the vacuole amounted to 33·8. If bound water is neglected the percentage amounts to 31·7. These values compare (see Table I) with 31·1 per cent. from chlorine and 28·3 per cent. from potassium. Thus both methods agree in giving values of about 30 per cent. The agreement between the two methods suggests that the volume¹ of protoplasm in the leaf is much greater than is generally supposed.

¹ The volume of the protoplasm is assumed not to differ greatly from the volume of water released on its decomposition.

Thus far we have not found great variation in this value. We have used the method of calculation from the concentrations in the saps as it is more convenient and probably more accurate. The values calculated from potassium



TEXT-FIG. 1. Weights of water in sap, expressed at different pressures, from 100 gm. of leaf material.

are usually somewhat less than those calculated from chlorine. In Table III we show the results for leaves of three different ages on the main axis of the cotton plant. It will be observed that the values are somewhat greater for the old leaves.

TABLE III

*Percentages of Total Water (sap) in Vacuole of Leaves of Three Different Ages.
Calculations made from Concentrations of Chlorine and Potassium in the Saps*

		From chlorine.	From potassium.
Top leaves	.	27·4	25·6
Middle leaves	.	30·2	25·8
Bottom leaves	.	35·4	37·0
Sig. diff. $P = 0\cdot05$.	6·5	11·6
Sig. diff. $P = 0\cdot10$.	5·6	10·0

As the proportion of the total water and the concentration of solutes in the vacuole are both small, it follows that the percentage of the total *solutes* present in the vacuole is also small. Thus, for soluble potassium (see Table I) only 6·9 per cent. was dissolved in the vacuolar sap, the balance being presumably in solution in the protoplasm.

III. RESPIRATION BY THE RESIDUE

Probably the most remarkable feature of these experiments is that pressures in the hydraulic press of 14,000 lb. per sq. in. are unable to express the water and solutes in the protoplasm and that gentle rubbing of the residue between the fingers and thumb permits of their easy expression. Freezing, boiling, or treatment of the residue by anaesthetics also of course allows of easy expression of the protoplasmic sap. It seems clear that the protoplasm possesses a gross structure that is destroyed by small shearing forces, but which is able to withstand relatively large direct pressures. Destruction of this structure leads to death and release of the protoplasmic water and solutes. It ought to follow therefore, that the residue from the hydraulic press should be able to respire, and that after it has been gently rubbed it should cease to do so. Gentle stroking of the surface of the leaf, as Audus (1935) has shown, may actually increase respiration. The rubbing of the residue between the fingers and the thumb might, therefore, be expected to increase respiration even though such increase might be very temporary. The following experiment was designed to find out whether respiratory activity is shown by the residue both before and after it is rubbed between the fingers and the thumb.

There were three treatments and two samples for each treatment. Six samples each of 50 gm. of leaf material were prepared as if for pressing in the hydraulic press and were treated as follows:

A. Normal samples.

1. Carbon dioxide content determined by the alcohol method (Willaman and Brown, 1930).
2. Placed in respiration chamber and carbon dioxide output determined for two hours. Sample then removed from chamber and carbon dioxide content of tissue determined by the alcohol method.

B. Pressed residue samples.

3. Sap extracted in hydraulic press under pressure of 14,000 lb. per sq. in.; residue and sap mixed and carbon dioxide content determined by the alcohol method.
4. Sap extracted in hydraulic press under pressure of 14,000 lb. per sq. in.; residue loosened and mixed with sap. Placed in respiration chamber and carbon dioxide output determined for two hours. Sample then removed from chamber and carbon dioxide content of tissue determined by the alcohol method.

C. Pressed residue samples rubbed between fingers and thumb.

5. Treatment as for sample 3, but residue also rubbed between fingers and thumb before determination of carbon dioxide content.
6. Treatment as for sample 4, but residue also rubbed between fingers and thumb before being placed in respiration chamber.

TABLE IV

Carbon Dioxide (mg. per 50 gm. fresh weight)

Treatment.	A. Normal.	B. Residue + sap.	C. Finger-rubbed residue, + sap.
Originally present . . .	24·8	17·1	2·3
Minutes in (0- 30 . . .	37·7	40·4	—
respiration (30- 60 . . .	29·8	34·0	—
chamber (60- 90 . . .	32·2	26·1	2·3
90-120 . . .	28·9	27·0	—
After removal from chamber . . .	25·4	14·0	1·0
Total produced during 2 hour period . . .	129·2	124·4	1·0

The results are shown in Table IV. It will be noticed that the carbon dioxide content for each of the three treatments was determined at the beginning and at the end of the experiment. Inspection of the table will make it clear that exposure of the leaf to pressures of 14,000 lb. per sq. in. in the hydraulic press has not stopped respiration and that rubbing the residue between the fingers and the thumb has brought it to a standstill. As far as can be judged, respiration was as active in the residue as in the normal samples. It seems to have been less active in the second than in the first hour.

IV. IMBIBITION BY THE RESIDUE

After the leaves have been subjected to pressures of 14,000 lb. per sq. in. in the hydraulic press, there are no air spaces to be seen. Under the microscope a section mounted in liquid paraffin shows a compacted mass of cells. Photographs of sections of a leaf before and after pressing are shown in Pl. XVII. When the compact residue is placed in water it swells with great rapidity and the flaccid residue becomes turgid. A section of the same leaf

after being pressed and immersed in water is also shown in Pl. XVII. The sections were cut freehand and rather thick in order to show the proportions of the tissues with as little disturbance as possible. The magnification is the same for the three photographs. It will be seen that recovery appears to be complete and that in place of an irregular and compacted mass of tissue the individual tissues can easily be distinguished. The thickness of the residue is only about 55 per cent. of that of the unpressed leaf, and that of the latter is indistinguishable from that of the residue after immersion in water. The cells, however, appear to be quite homogeneous and without a large central vacuole. In this connexion it is of interest to note that Hartmair (1937) has found that wounding certain cells followed by treatment by neutral red in conductivity water leads to a marked contraction of the vacuole and equivalent extension of the protoplasm.

In an attempt to determine more accurately whether the leaf regains its original thickness, the distance between the dorsal and ventral surfaces was measured in the microscope in three sections, mounted in liquid paraffin, from each of five leaves : (1) before the application of pressure, (2) after the expression of the vacuolar sap in the press, and (3) after the immersion of the residue in water. The mean measurements for each leaf are shown in Table V.

It will be noticed that the sampling variation is considerable. There does, however, appear to have been considerable recovery after immersion of the residue in water. The mean thickness of the residue was 0.519 mm. and that of the normal leaf and immersed residue 0.658 mm. The thickness of the residue was 79 per cent. of that of the unpressed leaf; this value is much greater than that of the leaf shown in Pl. XVII. The thickness of the residue in the press while under pressure is probably somewhat less than when the pressure is removed and the section mounted in liquid paraffin. The pressure in the centre of the wad will also be greater than that at the periphery. Calculation of the volume of air-spaces from the volume of the sap expressed, and the change in volume under pressure is not of course feasible.

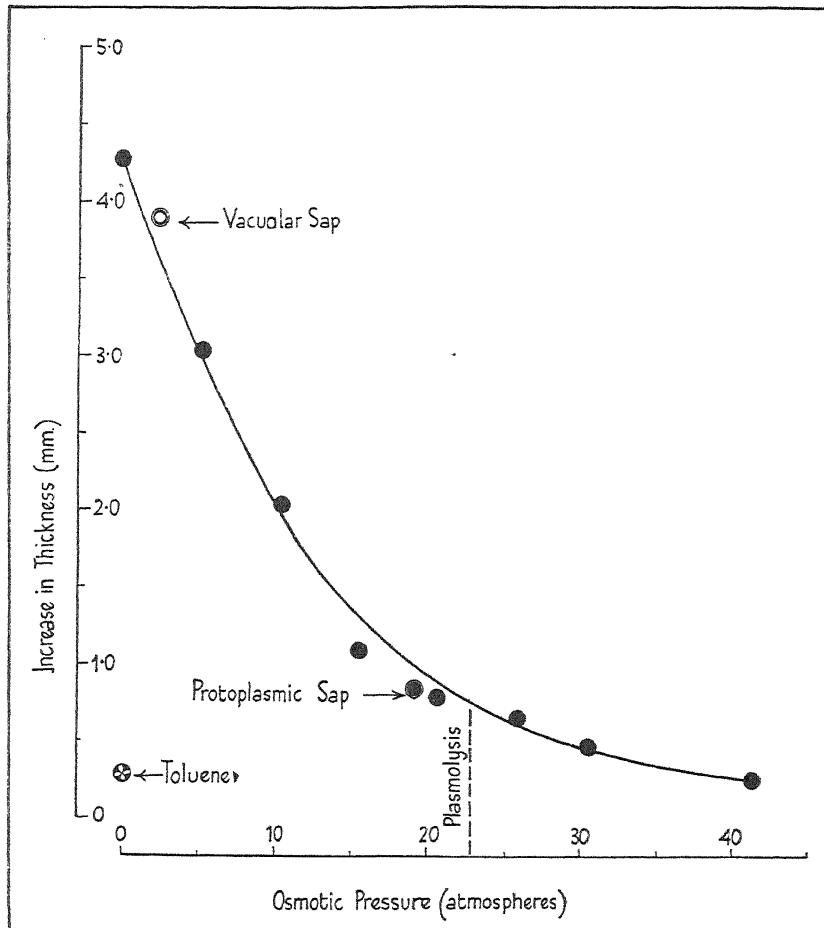
TABLE V
Thickness (mm.) of Leaves

Leaf No.	Normal.	Residue.	Residue after immersion in water.
1	0.676	0.511	0.620
2	0.667	0.589	0.651
3	0.625	0.505	0.640
4	0.629	0.536	0.703
5	0.692	0.453	0.676
Mean	0.658	0.519	0.658

Sig. diff. ($P = 0.05$) = 0.068

We have endeavoured to find out the extent to which swelling of the residue occurs in (1) sucrose solutions of varying osmotic pressure, and (2) in the vacuolar and protoplasmic (residue) saps after their expression from the

leaf. The procedure adopted was as follows: The leaves were pressed in the hydraulic press in the usual way. In the central part of the wad the pressure of course exceeds that at the periphery and the percentage of sap expressed is



TEXT-FIG. 2. Increase in thickness of leaf residue in sucrose solutions of varying osmotic pressures, in vacuolar and protoplasmic saps and in toluene-water. Osmotic pressure of plasmolysing solution at incipient plasmolysis of unpressed leaf shown by broken line.

therefore somewhat greater. In this central part of the wad cylinders of residue were cut out with a cork-borer of 1 cm. diameter. These cylinders were then placed in the solutions of varying osmotic pressures and in the two saps (i.e. vacuolar and protoplasmic), and their increase in thickness observed with the aid of a magnifying lever. The osmotic pressures of the saps were determined cryoscopically. Two cylinders were used for each solution and for each of the saps. The mean results are shown graphically in Text-fig. 2.

The thickness of the cylinders (residue) was approximately 7.5 mm. and (see Text-fig. 2) the increase in thickness in water 4.28 mm. The thickness of the residue before immersion was therefore 63.7 per cent. of that after immersion. Cylinders placed in toluene-water showed very little (3.7 per cent.) expansion. We have found in other experiments that cylinders from material frozen in carbon dioxide may also show about the same amount of swelling. Expansion in excess of that shown by the toluene-treated cylinders is presumably due in some way to the living protoplasm. In this experiment, as the imbibed residue did not show vacuolation, it might be assumed that we are here dealing with colloidal imbibition (cf. MacDougal, 1920). As, however, very nearly the original volume of the tissue appears to be regained, and as the cells before the application of pressure are vacuolated, a metabolic factor in the uptake of water is perhaps suggested. It will be observed that considerable swelling occurred in solutions of much higher osmotic pressure than that of the vacuolar sap and that some expansion even occurred in the protoplasmic sap. The expansion in the two saps was what would be expected from their respective osmotic pressures. It will be noted that the osmotic pressure of the external solution at incipient plasmolysis¹ (see Text-fig. 2) of the fresh leaves (i.e. before the application of pressure) used in this experiment even exceeded that of the protoplasmic sap, though it is doubtful whether the difference can be regarded as significant. In other experiments, however, this difference has been fully significant and is, it may be noted, always in the same direction.

It is difficult to explain all these results on the osmotic theory of cell-water relations, and it will be best to postpone their discussion till the next section.

V. DISCUSSION

It is often assumed that in vacuolated cells the protoplasm is restricted to a thin film between an enormous vacuole and the cell wall (cf. Barnes, 1937). According to this view, the volume of the protoplasm is too small to play any important part in water absorption, and it is therefore regarded only as a semi-permeable membrane permitting the vacuole to control water absorption by osmotic processes (cf. Stiles, 1936).

While this view of the relation between the volumes of vacuole and protoplasm may be true of plants that live in water, it is doubtful if it is generally true of land plants. Although Sinnott and Trombetta (1936) concluded that 'the problem of determining cytoplasmic volume in plant cells seems almost a hopeless one', there are a few measurements recorded in the literature. Thus, Ilijin's (1934) measurements on the leaf epidermis of *Hedera helix*

¹ Strips (5.0×1.0 mm.) were cut from four leaves. These were placed in sucrose solutions differing in concentration by approximately 0.1 mol. and injected by evacuation. After six hours immersion, sections were cut and mounted for examination in their respective solutions. The results were as follows:

Solution of osmotic pressure 20.7 atmospheres —No plasmolysis.

" " 22.8 —All the epidermal and spongy mesophyll cells plasmolysed. In the palisade tissue a few cells were plasmolysed but most were normal.

showed that the vacuole occupies only 38 per cent. of the cell volume, while in the mesophyll he found that it occupied about 48 per cent. Scarth and Levitt (1937) record values for the percentage of the cell area occupied by protoplasm in the cortical cells of apple twigs. The values ranged from 42·2 to 57·6.

Our (Phillis and Mason, 1937) own measurements indicated 'that nearly half the volume of the mesophyll cells in cotton, excluding the cells' walls, consists of cytoplasm'. It is not an easy matter to determine the volume relations of cell-wall, protoplasm, and vacuole for the whole lamina, especially in green leaves, for of course no decolorizing agent can be used. Our measurements were for this reason made on the white portions of variegated leaves which may, of course, differ from the green parts. We have found that the tonoplast is revealed with great distinctness with the Ultropaque microscope. It is not apparently a reflection image, for the granular protoplasm can be distinctly seen as far as the tonoplast. The protoplasm shows up particularly well in the cortical cells of the root. Pl. XVII, Figs. 4 and 5, shows photographs of such cells, from longitudinal sections about $1\frac{1}{2}$ in. behind the root-tip, taken with the aid of the Ultropaque microscope. The granular protoplasm shows brightly, while the vacuoles remain dark. Proof that we are really dealing with protoplasm and not a reflection image has been furnished by the observation of protoplasmic streaming in such cells.

In the present paper our *calculations* suggest that in the leaf the vacuole may occupy only about 30 per cent. of the volume of the cell. This of course at the best can only be a rough approximation as the volume relations of water and the vitaid molecule are not known and are no doubt variable. The problem is further complicated by the presence of bound water, or rather by what the chlorine method (cf. Mason and Phillis, 1936) suggests as being present in the form of bound water. Strictly speaking, the water united with the vitaid molecule should be regarded as bound water for it is probably not itself functioning as a solvent. The view that the protoplasm occupies too small a proportion of the cell to account for water absorption may thus be founded on a misconception, for it would appear that the volume of the protoplasm may actually exceed that of the vacuole.

Bennet-Clark, Greenwood, and Barker (1936) have recently questioned on other grounds the view that the protoplast functions merely as a semi-permeable membrane and takes no active (metabolic) part in water absorption. They have shown 'that the "osmotic value" of the cell sap as determined by the plasmolytic method is markedly greater than the osmotic pressure of the cell sap as determined by the cryoscopic method in certain tissues, but in certain other tissues the two may be equal'. They also found that the former were not plasmolysed by their own expressed sap, whereas in the case of the latter approximately half of the cells were plasmolysed. They concluded that in the former there was active secretion of water into the vacuole. In their terminology, the *gross water absorption pressure* of the cell at limiting

plasmolysis ('osmotic value' at incipient plasmolysis) is equal to the sum of the *osmotic pressure* of the vacuolar sap and the *secretion pressure* driving water into the vacuole.

Bennet-Clark, Greenwood, and Barker assumed the osmotic pressure of the cell to be that of the sap extracted from the tissue after freezing. Our results suggest, at least in the case of cotton, that the osmotic pressure of the vacuole may be much less than that of sap derived from the protoplasm and also of course less than that of the sap extracted from the cell. The values for the leaves used in obtaining the results given in Text-fig. 2 are shown in Table VI.

TABLE VI

*Osmotic Pressure (atmospheres) of Saps from Vacuole and Protoplasm
and of Plasmolysing Solution*

Vacuole	:	:	:	:	2·4
Protoplasm	:	:	:	:	19·3
Plasmolysing solution	:	:	:	:	22·8

If our conclusions concerning the distribution of water in the cell have any basis in fact, it seems evident that the secretion pressure (22·8–2·4, i.e. 20·4 atmospheres) in these leaves was much greater than anything contemplated by Bennet-Clark, Greenwood, and Barker. The results shown in Text-fig. 2 suggest that this may actually be so and that there are very large imbibitional pressures in the protoplasm. It is true it has not yet been demonstrated that these imbibitional pressures are dependent on a supply of metabolic energy or that they are accompanied by the secretion of water to form vacuoles. It seems clear, however, that they greatly exceed the osmotic pressure of the vacuole and it may be surmised that they are not unrelated to the secretion pressure postulated by Bennet-Clark, Greenwood, and Barker. The water absorption pressure of the residue would appear in this experiment (see Text-fig. 2) to have been somewhat greater than the osmotic pressure of the sucrose solution which caused plasmolysis of the unpressed, normal leaf.

Guttation from the surfaces of fungi, hydathodes, and tips of grasses has long been known. The mechanism of this secretion of water from the surface is still uncertain (cf. Blackman, 1921). Dixon (1938) has shown that even the mesophyll cells of the leaf can secrete water against a vapour-pressure gradient, and that oxygen is required for the process. The suggestion of Bennet-Clark, Greenwood, and Barker that water is secreted internally into the vacuole is something new. This internal secretion would seem to be akin to the secretion that takes place from the periphery of the contractile vacuole, which has been shown to depend on respiratory energy (cf. Barnes, 1937). Whether external secretion (i.e. guttation) from the surface of the cell is due to the vacuolar contents being forced by internal secretion pressures through fissures in the protoplasm in the same way that seems to occur when pressure is applied to tissues in the hydraulic press is a question that cannot at present

be answered. White (1938) has shown that very high pressure can be developed in the endodermal cylinder of the root.

VI. SUMMARY

1. A method is described for the separation of the sap in the vacuole from the sap derived from the decomposition of the protoplasm. From the concentrations in these saps and the concentration in sap from the whole cell, the distribution of water between the vacuole and the protoplasm was calculated. The distribution of sap-water in the cell was also estimated by extrapolation of the curve relating pressure in the hydraulic press and weight of sap-water obtained. Both methods indicated that only about 30 per cent. of the water in the cell is present in the vacuole. It is pointed out that these results do not accord with the view that the protoplasm occupies too small a proportion of the cell to exercise an important part in regulating the absorption of water.

2. It is shown that the osmotic pressure of the vacuolar sap is very much less than that of the solution required to plasmolysate the mesophyll. It is also demonstrated that the compact leaf residue from the hydraulic press after the vacuolar sap has been extracted exhibits (1) respiratory activity, and (2) can absorb water from solutions of much greater osmotic pressures than that of the vacuolar sap and also from the vacuolar sap itself. These observations are considered to support the suggestion of Bennet-Clark, Greenwood, and Barker that water is secreted into the vacuole by the protoplasm.

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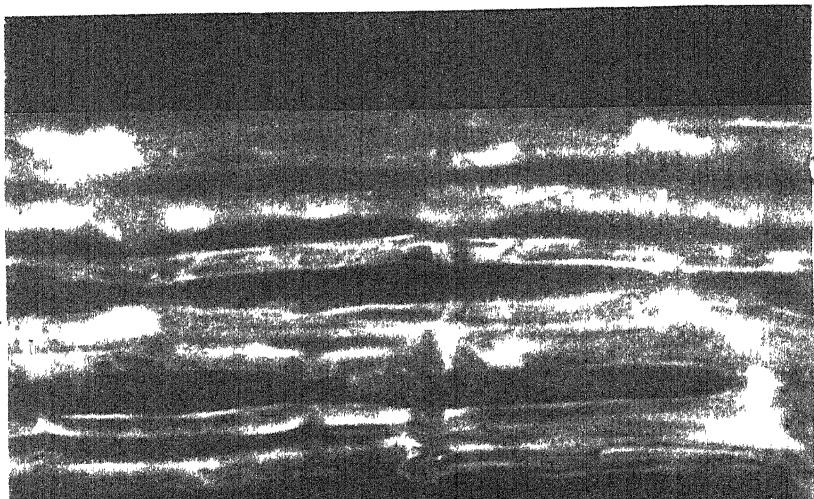
EXPLANATION OF PLATE XVII

Illustrating Messrs. Mason and Phllis's paper on 'Experiments on the Extraction of Sap from the Vacuole of the Leaf of the Cotton Plant and their Bearing on the Osmotic Theory of Water Absorption by the Cells.'

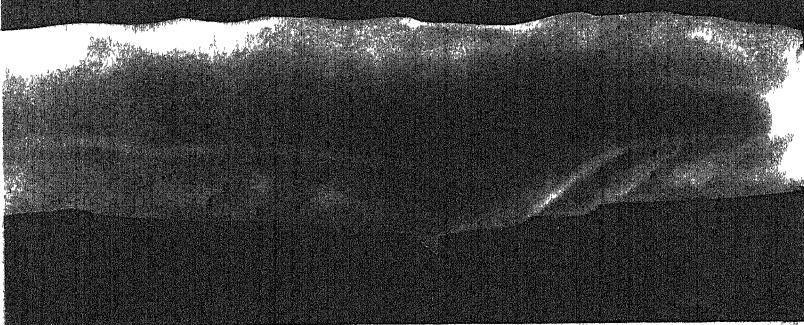
Figs. 1-3. Sections of cotton leaf: Fig. 1. Before pressure. Fig. 2. After pressure. Fig. 3. After pressure and immersion in water. $\times 80$.

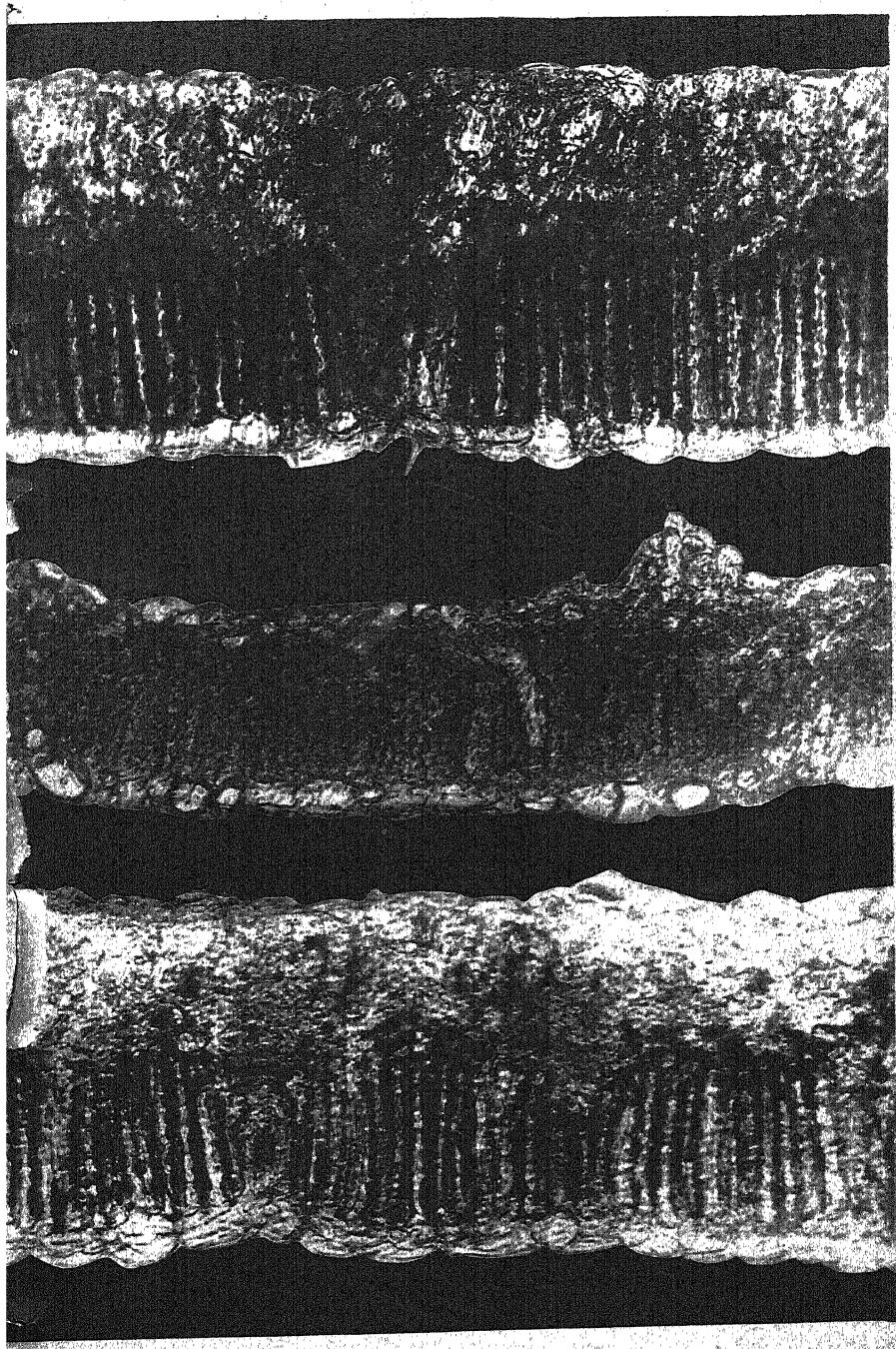
Figs. 4 and 5. Sections of root cortex showing relative dimensions of protoplasm and vacuole (Taken with Leitz Ultropaque objective U— $0.23 \times W$). Fig. 4. $\times 60$. Fig. 5. $\times 120$.

4



5





Chromosome Structure and the Mechanics of Mitosis and Meiosis

I. Mitosis in *Lilium*¹

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With Plates XVIII and XIX

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I. INTRODUCTION

THE classical researches of Morgan and his co-workers have placed the chromosome theory of heredity on a firm basis. But cytological evidence in support of these genetical studies has not yet passed the stage of speculation. Divergent views are still current regarding the internal structure of the chromosomes, the time and mode of their division, and the cytological mechanism of crossing over. With the magnifications now possible with our high-power microscopes the finer details of internal structure of the chromosomes in living nuclei are still beyond correct resolution. We have therefore to examine materials that have been treated with suitable reagents and compare these observations with those of other organisms at the same stage, treated similarly as well as with a variety of reagents. New and improved cytological methods have enabled us to approach this difficult study in a manner less open to the criticism to which it has been subjected in the past. Moreover, the investigations of Kuwada and other Japanese botanists on living nuclei under dark field illumination have not only clarified many obscure points in chromosome mechanics, but also confirmed in several details conclusions reached by the more orthodox methods.

¹ Material presented in this paper formed part of a thesis approved for the degree of Master of Science in the University of Madras.

The present investigation was undertaken with a view to examine the structure of chromosomes and correlate this structure with their behaviour in mitosis as well as meiosis. *Lilium* was selected as a suitable material as it possesses unusually large chromosomes. Although this genus has been extensively used for various cytological investigations, very little work has yet been done on the structure of its chromosomes. The present paper deals with the structure and division of the somatic chromosomes in *Lilium*. Similar studies on the meiotic chromosomes will form the subject of a subsequent paper.

II. MATERIAL AND METHODS

The present investigation is limited to three species of *Lilium*: *L. neilgherrense*, *L. Henryi*, and *L. tigrinum*.

Lilium neilgherrense Wight is a plant growing on the higher altitudes of south India. The material for this study was collected from Kodaikanal, a hill station in south India. The cytology of this species has not been studied hitherto, and as its chromosomes appeared very favourable for a critical examination of their internal structure, extensive collection of material was made in June of 1935 and 1936. The other species are garden plants collected at Kodaikanal through the courtesy of Mr. M. Maiden and Dr. F. H. Gravely. The structure of chromosomes has been studied mainly from sections of young ovaries, anthers, and root-tips of *L. neilgherrense*, while the other species were used for purposes of comparison only.

Root-tips and young ovaries were fixed in the following standard fluids and their modifications: Flemming weak and medium, Hermann's, Merkel's, Bouin's, La Cour's 2BE and 2BD, Smith's S₁ and S₂, Taylor's and Navashin's fluids. Time for fixation varied from twelve to twenty-four hours. For the fixation of root-tips Sharp's (1929) 'method twenty' and Nebel's (1933) method were tried with success. Most of the preparations were made according to La Cour's (1931) schedule, and these were equally satisfactory in regard to internal structure of chromosomes. An exhaust pump was used to facilitate rapid penetration of the fluid. Washing was done in running water for more than six hours and then completed with three or four changes of tepid water. Dehydration was done in alcohol through a close series starting with 2½ per cent. Microtome sections varying from 6 to 40 μ thickness were cut and stained in iodine-gentian violet.

Young ovaries for the study of somatic chromosome structure and embryo-sac development were fixed after trimming the sides of the ovaries with a sharp blade and then treating them in Carnoy's fluid or 20 per cent. ethyl alcohol. They were then cut into 2 mm. thick pieces before transferring to the fixative. Some of the ovaries thus treated were left in Navashin's fluid for two to four days. On staining with gentian violet it was observed that material treated in this manner showed more rapid and clearer differentiation in clove oil than the material treated by either Sharp's or

Nebel's methods. Very clear preparations showing chromonema spirals were thus obtained.

III. NUMBER AND MORPHOLOGY OF THE CHROMOSOMES OF *LILIUM NEILGHERRENSE*

The diploid number of chromosomes in this species of *Lilium* has been determined for the first time as twenty-four. Pl. XIX, Fig. 20, shows a metaphase plate from a root-tip cell showing twenty-four chromosomes. Slight variations in the relative lengths of chromosomes have been observed in materials collected from different localities in Kodaikanal. In some of the root-tips examined, a fragment chromosome was seen. In Pl. XIX, Fig. 20, two chromosomes show secondary constrictions, though this feature was not observed in materials from which oogenesis and meiosis were studied. Except for these minor structural variations, the morphology of the twenty-four chromosomes is similar in materials collected from different localities. In Pl. XVIII, Fig. 19, the haploid complement of twelve chromosomes, seen at the metaphase of the first post-meiotic mitosis at the micropylar end in the embryo-sac, is drawn seriatum to show the comparative size and morphology of the chromosomes. There are two sub-median attachment chromosomes denoted by the letters S₁ and S₂, one J chromosome, and nine sub-terminal attachment chromosomes denoted by the letters A to I. The length of the chromosomes varies from 8.5 μ to 16.5 μ , while in root-tip mitosis the variation is between 12.5 μ and 25 μ . In oogenesis the organization of a triploid complement was seen at the chalazal end of the embryo-sac in the second post-meiotic mitosis as observed by Cooper (1935) in other species of *Lilium*. Three each of the S₁, S₂, and J chromosomes are seen more prominently than the rest (Pl. XVIII, Fig. 18).

IV. DESCRIPTION OF SOMATIC DIVISION

After the separation of the chromosome halves at late metaphase, each group of daughter chromosomes moves towards either pole. In deeply-stained preparations, as well as in those treated with certain fixatives, their internal structure is obscure. But in material treated with Benda's fixative and properly differentiated they show a clear spiral structure. This is most distinctly seen in those fixed in Navashin's fluid and treated as per schedule given above. Each anaphase chromosome shows two spiral chromonemata embedded in a common matrix (Pl. XVIII, Fig. 1). The structure and relation of the two spirals of each anaphase chromosome were studied very critically owing to their importance in the interpretation of subsequent stages and the mode of spiralization of each chromonema. Pl. XVIII, Fig. 1, shows an anaphase group in which the two coiled chromonemata are very distinct in each chromosome (see photomicrograph, Fig. 22). In most of these the two coils are entirely free from each other and run parallel, while in a few the two coils are twisted on each other. The degree of intertwining of the two spirals of

each chromosome varies to some extent in different cells, and the crossing-points of the two spirals range from one to four. Pl. XVIII, Fig. 2, shows anaphase chromosomes drawn on a higher magnification. In these the spiral structure is brought out more clearly.

When the chromosomes present a side view in the optical field, due to the superposing of one chromonema spiral on another, a false appearance of intertwining at every turn of the spiral is seen. This false appearance of intertwining is, however, characteristically different from the true intertwining shown in Figs. 2 *a*, *b*, and *c*. Kuwada and Nakamura (1933) have shown that when in a double spiral the component spirals are separated from each other and are shifted in the direction of their longitudinal axis, they will give the false appearance of two chords twisted about each other. This is very well illustrated by photographs of wire models in their Text-fig. 1. In deeply stained preparations such parallel approximation of two spirals would give a moniliform appearance, so frequently reported in anaphase chromosomes.

In some chromosomes, particularly those of root-tip nuclei, the chromonemata exist in some regions as an apparently single spiral thread, while along other regions of the same chromosome the duality is clear. Nebel (1933), in his study of the chromosomes in the root-tips of *Tradescantia*, has found the anaphase chromosomes as having two free spiral chromonemata pushed into one another.

In a few cases, shown in Pl. XVIII, Fig. 2 *c*, the chromosomes showed very deeply chromatic borders with a few twists, but here the chromonema spirals are not visible. This parallel alinement of chromatic halves of anaphase chromosomes, without any suggestion of internal spiral structure, has been illustrated by Sharp (1929), Hedayetullah (1931), Perry (1932), and Huskins and Hunter (1935). The number of twists increases as the anaphase progresses (cf. Hedayetullah, 1931, and Perry, 1932).

Tassement polaire. It has been reported for several plants that the end of anaphase is marked by a clumping together of the chromosomes to such a degree as to obscure both their individuality as well as their internal structure. Grégoire, who first described this appearance, gave it the name 'tassement polaire'. In *Lilium*, though the clumping has been frequently observed in somatic mitosis, the individuality of most of the chromosomes and their internal structure could be clearly made out in sufficiently de-stained preparations (Pl. XVIII, Fig. 3). In Pl. XVIII, Fig. 4, the late 'tassement polaire' condition is shown. Duality and twisted aspect are particularly clear in the projecting arms of two chromosomes. The 'tassement polaire' clumping involves a maximum contraction of the chromosomes in length. From the zigzag or spiral, which the chromosome as a whole is forced to assume, it appears that the matrix substance contracts to a greater extent and that the more chromatic portion of the chromosome is less affected by it. But even under identical conditions of fixation, no polar clumping is observed at the meiotic telophase,

and so it is unlikely that 'tassemement polaire' is a mere artefact of fixation as Overton (1922) has assumed, though the clumping may be accentuated by fixation. The stage from the late anaphase to the completion of a nuclear membrane may be considered as the most delicate with reference to its reaction to fixatives.

Telophase. A thin nuclear membrane becomes distinct after the emergence of the chromosomes from the 'tassemement polaire'. At this stage a septum is formed in the middle of the cell and two daughter-cells are thus organized. The nucleus then assumes a more central position.

At the early telophase the nucleus is kidney-shaped, but as the telophase progresses the nucleus enlarges in size and assumes a more regular shape. It is difficult to distinguish early telophase chromosomes and their structure. But by mid-telophase (Pl. XVIII, Fig. 5) the structure of each chromosome becomes more clearly visible. Each chromosome is now seen to contain two spiral chromonemata which are closely associated at certain regions. The more significant telophase alterations which the chromosomes undergo are an elongation of the chromosomes and a relaxation of the anaphase spirals after their maximum contraction at 'tassemement polaire'. At mid-telophase (Pl. XVIII, Fig. 5), in chromosomes where duality has not been completely obscured by the close association of the two spiral chromonemata, it is found that the chromonemata are twisted on each other, the twists being more than were observed at the anaphase. Pl. XVIII, Fig. 6 *a-c*, show the twisted dual aspect as well as regions where the chromonemata are so closely associated as to appear as a single spiral. In Pl. XVIII, Fig. 7, the late telophase condition is shown. The nucleus has assumed a regular shape and the chromosomes are seen as apparently single stranded spirals. Hsu Siang (1932) has figured the telophase chromosomes in *Lilium tigrinum* as having two chromatids twisted on each other at three or four points, while one chromosome shows only a half-twist (see his Fig. 19). A comparison of this with his figures of anaphase chromosomes shows that there is a reduction in the number of twists subsequent to anaphase. In *L. neilgherrense*, however, there is an increase in the number of twists during telophasic changes. Hedayetullah (1931) noted a significant difference in the structure of chromosomes at telophase in materials treated with Merkel's fluid and Flemming's or Hermann's fluids. In those of the former he found parallel duality with chromomeric appearance and occasionally one or two twists, while in those of the latter the two threads of a chromosome appeared uniform and intertwined at several regions. No such difference was noted in *Lilium*, though the spiral structure and duality of chromosomes were brought out clearly by certain fixatives while certain others obscured them. In *Lilium*, anastomoses or interchromosomal connexions were seen in the telophase nuclei in materials treated with osmic fixatives. In those subjected to the long schedule of Navashin fixation and de-stained to show the internal structure, the chromonemata alone were visible at this stage, and no interchromosomal connexions were seen. Since

it is found that prolonged treatment with Navashin's fluid has a solvent effect on the matrix alone, it is likely that the so-called anastomoses are merely strands of achromatic matrix connecting adjacent chromosomes. Telezynsky (1930) has recorded the presence of lateral anastomoses in living material both in the telophase and the prophase. Sarbadhikari (1927) and a few others interpret them as outgrowths from the body of the chromosomes. Kaufmann (1926) considers them to be outgrowths of highly active, mobile chromatic threads.

Interphase. The nucleus that passes on from telophase to a state of inactivity as regards visible chromosome behaviour has been described as entering a resting stage. In rapidly-dividing tissues, like the meristems of root-tips, the interphase between the telophase and the succeeding prophase may be expected to be of very short duration, while in tissues where nuclear divisions are less frequent this stage may be more prolonged. In these latter nuclei, which have apparently remained in the 'resting stage' for a longer period, the chromatic tracts are very difficult to follow. The nucleus at certain regions shows the presence of discontinuous chromatic dots, which sometimes show a spiral course, while at other regions faintly stained chromatic spirals, which are continuous for short lengths, could be made out. In the nucleus which is apparently emerging from the interphase (Pl. XVIII, Fig. 8) or has remained in that condition for only a short period, continuous portions of the chromosomes are more distinctly seen and the chromatic dots are less prominent. The karyolymph also retains the stain to a certain extent. The spiral organization of the chromosomes is distinct at several regions (Pl. XVIII, Fig. 8). Thus it is seen that at the interphase also the spiral structure is maintained as at the preceding late telophase. No evidence of duality is visible in any chromosome at this stage.

Early prophase. This stage is marked by the reappearance of the chromatic tracts which became less distinct with the passage of the telophase nucleus into the interphase. The spirals shown in Pl. XVIII, Fig. 8, become more clear and the whole nucleus presents an appearance similar to the late telophase, with the difference that some of the chromosomes are run into zigzags. Darlington (1935) states that the chromosomes which appear at the early prophase do not possess the regular spirals seen at the telophase, as these spirals are considerably relaxed before entering interphase. In *Lilium* the telophase spirals are not very much relaxed before entering interphase, and even the interphase nucleus shows evidence of the presence of regular spirals. In rapidly-dividing tissues the nuclei that emerge from interphase are hardly distinguishable from those that enter it. One method of identifying an early prophase nucleus from a late telophase nucleus is afforded by the fact that while the two daughter-nuclei, produced as the result of a division, show nearly identical changes in their development and in the structure of their chromosomes from early telophase to the onset of interphase, the duration of interphase may vary with each nucleus, as the result of which they may enter the next prophase at different times. In the meristems of root-tips,

where very rapid succession of divisions takes place, there may be no real interphase, and some prophase nuclei may show even the straight arrangement of the chromosomes unaltered as in Sharp's (1929) Figs. 20 and 26. These need not then be interpreted as telophase as Darlington (1935) would have it. In Pl. XVIII, Fig. 9, of early prophase showing this aspect, no evidence of duality is seen in any chromosome, though in some preparations an uneven outline of the chromosome thread is evident. Pl. XVIII, Fig. 10, represents a later stage from a more deeply-stained preparation, showing the persisting telophase spiral (referred to hereafter as the major spiral). Duality is visible at several points along the length of the chromosome in this figure, but the outline of the chromosome is more or less smooth. Fig. 11 shows a nucleus at very nearly the same stage but is drawn from a more de-stained preparation. The chromosome duality is very marked. The two chromatids are seen twisted on each other, and the number of twists is more than what was observed at anaphase, though the chromatids are not twisted at every region where they are seen to cross each other. The chromosomes at this stage have been described by some recent authors, Koshy (1933), Hoare (1934), and Gregory (1935), as composed of two tightly twisted chromonemata with a very large number of twists. While there is the probability that such differences in the degree of intertwining of chromatids may be expected in different genera, Sharp's (1929) observations on the structure of large somatic chromosomes of several plants is significant. He has shown in his figures only a few twists between chromatids, as is observed in the present study. He has further stated that 'careful examination of the points at which the two threads cross each other strongly suggests that they are not simply intertwined, but that their relative position is such as would permit them to move apart to two sides of the enveloping matrix without becoming entangled'.

In Pl. XVIII, Fig. 11, each chromatid is seen organized into a very regular spiral of small gyres. At certain regions it is very clear while along portions of the chromosome, where duality is not visible, only a regular corrugation of the chromatid is seen. This latter aspect might give the false impression that the chromatids are tightly twisted on each other (right side, bottom chromosome in Fig. 11). The chromosomes appear thicker and shorter than they were at an earlier stage of prophase, and the chromosomes are less crowded. This contraction of the chromosomes at the prophase of somatic mitosis has been recorded by several authors. Kaufmann (1931), Koshy (1933), and Hoare (1934) have described the prophasic contraction as being due to a new spiralization in each chromatid at this stage, though they did not actually observe this spiral at any stage prior to metaphase. Kuwada and Nakamura (1934) observed this spiral formation in the living nuclei of *Tradescantia*, though they have not illustrated it. Darlington (1935) states: 'The process of spiral formation during shortening (of the chromosome) has not been observed but comparison of the chromosome before and after shows that the two processes occur together.' Hedayetullah (1931) and Perry (1932)

observed only chromomeres in each chromatid at early prophase. Sharp (1929) has figured the uneven outlines of the mid-prophase chromosomes but has described it as due to the presence of chromomeres, though the chromomeric appearance is not so evident as in Hedayetullah's figures. Very clear evidence has been obtained regarding this stage, and it is seen that the corrugation in each chromatid of the early prophase marks the beginning of visibility of a new spiral which becomes increasingly clear from mid-prophase onwards (Pl. XVIII, Figs. 12, 13, and 14).

Mid-prophase. Concurrent with the increase in size of the nucleus, but more rapid in its pace, proceeds the shortening of the chromosome length mainly due to spiralization. The joint effect of these two forces, viz. chromosome contraction and nuclear enlargement, is to bring about a gradual straightening of the major spirals. With the disappearance of the major spiral (Pl. XVIII, Fig. 13) the apparent as well as real twists are considerably reduced in number and only a very few twists persist at late prophase and still fewer at metaphase. The chromatids in each chromosome run very nearly parallel and the minor spiral in each is very distinct. The chromatids increase in thickness up to metaphase. This appears to be due to the increase in diameter of the gyres of the spirals as well as to the deposition of more chromatic material. Sharp (1929) has described an increase in length of the chromosome associated with its mid-prophase straightening. In *Lilium* it was not possible to detect any actual elongation of the chromosome as a whole, though an apparent increase may be seen, presumably due to a difference in the time relation between the processes of new internal spiralization and the straightening of the major spiral.

Before the disappearance of the nuclear membrane, in some of the chromosomes duality is evident in each chromatid (Pl. XVIII, Fig. 14—arrow marks). It was seen from an examination of the late telophase and early prophase chromosomes that when two chromatid spirals become closely associated the duality of the chromosome becomes almost a matter of inference. Conversely we might expect, though this need not necessarily be so, that when duality is seen at certain regions in a chromatid spiral these regions are not the only places where cleavage has occurred, but that it is probably double along the whole length. This expectation appears all the more plausible when we consider the fact that the chromatid in which duality is evident is coiled into a spiral of smaller dimensions than the telophase spirals or the major spirals of the early prophase.

Hsu Siang (1932) has reported the absence of a matrix substance in the prophase chromosomes in *Lilium tigrinum*. In my preparations the matrix is seen to stain only very faintly during mid- and late prophase, though the matrix of the chromatids takes the stain more deeply. It may be that after the division of each chromatid in preparation for the chromosome separation at anaphase, the common matrix of the two chromatids accumulates more and more around each chromatid.

Late prophase. This stage may be marked off from mid-prophase by a tendency exhibited by the chromosomes to recede from their peripheral disposition. Pl. XVIII, Fig. 15, shows some of the late prophase chromosomes soon after the disappearance of the nuclear membrane and the nucleolus. The chromatids are seen very deeply stained while the matrix is very lightly stained. The spiral structure of the chromatids is clear in most of the chromosomes, while the duality of the chromatids is distinct only in certain regions. In some cases duality of the chromatids is seen for a length included by two gyres of the chromatid spiral, and it is significant that the two chromonemata in each chromatid are free at these regions. In Sharp's (1929) Figs. 44 and 45, of late prophase in *Vicia* and *Podophyllum*, similar duality and spiral structure are shown.

No chromosome shows more than three or four twists between the chromatids at this stage. Contrary to similar observations of Sharp and several others, Hedeyetullah (1931) has reported an increase in the number of twists between the component chromatids from mid- to late prophase. An examination of his figures suggests the possibility of his having confused early and mid-prophases with mid- and late prophases by attaching too much significance to variation due to the action of different fixatives. Further, Sharp (1929) has shown that when chromosomes at late prophase are viewed from the side it may present a false appearance of the two strands being twisted on each other, and such error in observation is more likely in chromosomes like those of *Tradescantia*, which are not large enough to allow any definite conclusions to be made as regards the interrelationship of the chromatids.

The chromosomes shorten still further and tend to aggregate in the middle of the nuclear chamber. With the movement of the chromosomes to the equator of the spindle they pass on to metaphase.

Metaphase. The chromosomes in the equatorial plate have their attachment constrictions all pointing to the centre, though the arms of the chromosomes lie in different directions (Pl. XIX, Fig. 20). At early metaphase the comparative lengths of the different chromosomes could not be made out, as in the limited space available the chromosomes are too long to lie in one plane. But as contraction proceeds further, they come to lie more or less in one plane. This aspect is shown in Pl. XIX, Fig. 20, which is drawn from a deeply stained preparation of a root-tip fixed in Benda's fluid. The daughter chromosomes are distinctly formed and in some cases are twisted on each other, while in others they are parallel. Duality of daughter chromosomes is not seen in this, but less stained preparations show it. The clearest preparations showing quadruple structure and the spiral organization of each chromonema in the daughter chromosomes were obtained from young ovaries and anthers subjected to the long schedule with Navashin's fixative. In some of these the quadruple structure was seen with diagrammatic clearness along whole lengths of chromosomes (Pl. XVIII, Figs. 16 and 17). As the data concerning the interrelationship of the two chromonemata of each daughter chromosome

has important bearing on the interpretation of the spiral mechanism and mode of chromonema duplication, the structure of metaphase chromosomes was studied very critically.

Pl. XVIII, Fig. 16*a*, shows an early metaphase chromosome exhibiting duality in each chromatid. The two chromonemata (shown by arrow-marks) are free for a length including three gyres of the spiral. The chromonemata are much thinner than the chromatids. Figs. 16*b* and *c* show duality at the ends of chromatids. In Fig. 16*d* the chromonemata are seen as free and separate for the major length of the chromosome. In Fig. 16*e* the spiral nature of each chromonema and their free and nearly parallel arrangement are seen. Fig. 16*f* shows a rather exceptional feature in this material; while two twists are still retained between the daughter chromosomes in the longer arm of the J chromosome, shown in the figure, in one of them four twists are seen between the daughter chromonemata. The structure of the end half of the other daughter chromosome is not clear, but the duality is indicated by the forked end. In Pl. XVIII, Fig. 17, some of the chromosomes of a late metaphase are shown at a lower magnification than Fig. 16. Of the twenty-four chromosomes only fourteen are shown in the figure. The chromosomes at the sides have separated at the attachment region and show the early stages of anaphase separation.

V. DISCUSSION

(a) *Structure of the chromosome.*

Critical examination of both fixed and living material in recent years has well established the view that each chromosome consists of a coiled thread (chromonema) embedded in a less-chromatic matrix. Although the presence of a coiled thread in chromosome make-up was observed as early as 1882 by Baranetzky, no serious notice of this aspect was taken till 1926, when Kaufmann reported that the chromosomes of *Tradescantia* contain double spiral bands in all phases of somatic and meiotic divisions. As a result of the work of Kaufmann (1926–36), Sharp (1929, 1934), and Kuwada and Nakamura (1933–7), which have been confirmed and elaborated by several contemporary cytologists, we have no doubt now that each chromosome is composed of two coiled chromonemata embedded in a less chromatic matrix. Darlington (1935, 1937) and Huskins and Smith (1935) and some other investigators hold the view that at certain stages of the nuclear cycle the chromosomes are made up of linear rows of small chromatic units called chromomeres, identifiable by their characteristic size and definite position in the chromosome thread. Belling (1931), by a special technique elaborated by him, demonstrated the presence of an ultra-microscopic particle near the centre of each chromomere. He also counted the number of chromomeres in various Liliaceous genera and obtained values ranging roughly from 1,000 to 2,500. The existence of chromomeres as definite morphological units has, however, been denied by Grégoire (1906), Sharp (1929), Bolles Lee (1920), Smith (1932), O'Mara

(1933), Koshy (1933, 1934, 1937), Hoare (1934), Gregory (1935), Gates and Nandi (1935), Naithani (1937), Gates (1937), and others. It is sufficient to state here that while no evidence was available which would appear to throw light on the view of the existence of chromomeres as definite morphological units, the present study afforded convincing proof of the existence of coiled threads (chromonemata) in all phases of chromosome history. Certain problems of chromosome structure connected with the chromonema theory, particularly the mechanism of spiralization and the time and mode of chromonema duplication, are still very controversial, and are therefore discussed in detail in this paper.

(b) *Spiral structure and chromonema behaviour in mitosis.*

The supporters of the chromonema theory claim to have observed the spiral structure of the chromosomes at some or nearly all phases of the nuclear cycle. Even the adherents of the older 'alveolar' and chromomere theories of chromosome structure observed spiral organization of chromatic threads in some phases of the nuclear cycle though there have been considerable differences of opinion as to the mode of their origin.

At the earliest stage of prophase when the chromosomes could be seen distinctly they are seen as remaining in a spiral form. The two chromatids are so closely associated that duality of the chromosome is difficult to make out. And it was also seen that the spirals observed at the preceding telophase reappear without much alteration at the prophase. In view of a new visible spiralization in each chromatid at prophase, this persisting telophase spiral has been referred to as the 'major spiral'. At the early prophase in well-differentiated preparations the outline of the chromosome is not smooth, but appears corrugated. At a little later stage when the duality which became obscure at the preceding telophase reappears at the dual regions, in place of the corrugations a spiral structure is seen in each chromatid. By mid-prophase this 'minor spiral' becomes very distinct in each chromatid. By late prophase we find that the pitch of the spiral has increased and the number of gyres decreased, as a result of which the chromosomes appear very much shortened. Duality in each chromatid first became evident in favourable regions before late prophase.

How this spiral originates has now to be considered. The forces, internal or external, responsible for forcing the chromonemata into spirals could not be determined directly from a study of fixed materials. But when we get a clear picture of chromonema and chromosome division and correlate their external behaviour with their internal structure it is easy to show which is the more probable mode of spiralization.

The assumption of a spiral by a straight thread or of a new spiral within a pre-existing spiral could take place in one of two ways. The spiral can arise by the rotation of the two ends in opposite directions and without any internal compensating twists or spiral readjustments, the number of gyres of the spiral

corresponding to the number of rotations of the ends. If we make such a spiral with a copper wire by winding it round a rod and then take out the rod and pull the spiral out straight without allowing any relative rotation of the ends, it will be found that the copper wire is internally twisted in the same direction as the spiral was wound, the number of such complete twists being equal to the number of gyres of the spiral. Thus, this mode of spiral formation involves an internal twist which is directly proportional to the gyre number. This type of spiral may be termed 'unbalanced spiral'. The second method by which a spiral could be formed is by the ends remaining relatively fixed without undergoing any rotation as in the previous case. Then in the course of spiralization an internal reversed twisting takes place, the number of such twists being equal to the number of gyres of the spiral. If this spiral is pulled out straight without any relative rotation of the ends, it will be found that there are no internal twists as in the previous case. Here the twisting produced by spiralization is balanced by a compensating twist in the opposite direction at each gyre of the spiral. This type of spiral may be termed a 'balanced spiral' (see Pl. XIX, Fig. 21 A and B).

The problem is; which of the two types of spirals is formed when a chromonema spiralizes, and whether the one type could change to the other or be derived from it.

In the history of the chromosome it is the chromonema that is seen to maintain a regular permanence, and hence a chromosome division normally involves a division of its constituent chromonemata. On genetical grounds it is necessary to take the division as qualitatively equivalent, and cytological observations show that it is quantitatively identical as well. In a spirally coiled chromonema if a cleavage takes place along the longitudinal axis of the spiral the chromonema will be cut into bits. As the chromonema in *Lilium* is found to remain as a spiral throughout the mitotic cycle, and as it is seen as an unbroken structure, the cleavage in this could have been only along the longitudinal axis of the thread forming the spiral and not along the axis of the spiral itself, i.e. the course of cleavage could only be spiral and not straight.

If the cleavage is equational and along the course of the chromonema thread, then it would give rise in a spiral to two spirals comparable in all respects. They will have the same number of internal twists as the parent spiral and the direction of twists will also be the same. In the unbalanced spiral, since the twists are in one direction only and that in the direction of the coiling of the spiral, the daughter spirals will be interlocked once for every gyre of the parent spiral. But in the balanced spiral, since the twists are reversed in each gyre of the spiral, the daughter spirals too will have this compensating twist and hence there will be no interlocking which will hinder their separation (see Pl. XIX, Fig. 21 A and B).

Thus, basing our conclusions on what appear to be very reasonable assumptions regarding the mode of cleavage, we come to recognize in the inter-relationship of the daughter spirals the cardinal difference between the balanced

and unbalanced types of parent spirals. It follows that an examination of the relationship between the daughter spirals would enable us to say which manner of spiralization is really operative. At late prophase where spiral structure of the chromatid is very clear, it is seen that the two daughter chromonemata of each chromatid are free from each other where duality is distinct. At metaphase a distinct quadruple structure is observed and the two chromonemata approximate to the borders of the chromatid matrix and exist as two independent coils. Two entirely free parallel chromonema spirals have been observed in each daughter chromosome at metaphase and in each chromosome at early anaphase. Sharp (1929) in his study of the structure of large somatic chromosomes obtained clear evidence of a parallel association of the two chromatids in each daughter chromosome at metaphase in *Trillium* root-tips. His Figs. 52 and 53 show very clearly this aspect as well as suggestions of the spiral nature of each chromatid. Similar structures at somatic metaphase, without any indication of an internal spiral, have been observed by Hedayetullah (1931), Perry (1932) and Huskins and Hunter (1935). Thus it is seen that the daughter-spiral chromonemata produced by a split in the parent coil are free by metaphase and could separate without entangling.

In tracing the development of the minor spiral from very early prophase it is seen that duality in each chromatid becomes evident by mid-prophase though clearly seen only at late prophase. So it is improbable that cleavage could have been completely effected in each *chromatid* before early prophase. Thus it is seen that the cleavage would have taken place only after the early organization of the new chromatid spiral. The next point is how the daughter chromonemata spiralize, whether independently or together. The two chromatids in each chromosome were seen as more or less separated from each other and spiralizing independently as mid-prophase was reached, though at early prophase the first signs of spiralization were seen when the two chromatids were so closely associated as to obscure chromosome duality. But the spiralization of the two chromonemata in each chromatid is not comparable to this. In most cases we find the two chromonemata associated as closely as the two chromatids were at early prophase, and this condition persists up to early metaphase. If independent spiralization had begun in each chromonema simultaneous with, or soon after their organization, then the duality of the chromatids would have become more apparent before metaphase. Kuwada and Nakamura (1935, p. 318) have stated that 'if the split halves (of the chromatids) are still so intimately associated with each other that they are coiled into a single spiral, and if they are separated first later from each other when the coiling has proceeded to a considerable extent, they will appear twisted about each other in the later stages, as observed by many investigators'. The contrary is observed in *Lilium*, though the split halves are closely associated in a single spiral.

If an equational cleavage takes place along an unbalanced spiral or after cleavage the two threads spiralize together into such a spiral, they will be

interlocked once for every turn of the spiral. If after cleavage in such a spiral the two halves spiralize independently, even then the secondary spirals will not get completely free as some twists will still persist. No such subsequent spiralization is visible in somatic mitosis in *Lilium*. So, since the two daughter chromonemata can slip out from its common spiral, the chromonemata should be considered as two free and independent spirals from the time of their organization. The mechanism of the unbalanced spiral does not account for such a relationship between the daughter spirals. If an equational cleavage takes place along a balanced spiral or after cleavage in a thread the two threads together or independently coil into such spirals, they will be entirely free from interlocking or intertwining. It follows, then, that the balanced spiral mechanism satisfactorily accounts for the structures observed in *Lilium* chromosomes.

The above interpretations have been based to a certain extent on the observation that the chromonematic constituents of each chromosome are defined one mitotic cycle prior to their separation into daughter chromosomes. It would appear, however, that if we could have more convincing evidence in support of the contentions of Nebel (1933) or Goodspeed (1935) that the chromonemata divide two or three mitotic cycles prior to their separation into daughter chromosomes, then the evidence on which the balanced spiral mechanism was shown to account for the observations in *Lilium* may not alone be sufficient to disprove the probability of the unbalanced spiral also giving rise to the appearances described. We could dismiss such a probability if we consider for a moment the interpretations of these authors. Nebel (1933) has stated that at no stage in the mitotic cycle do chromonemata entangle with each other, and this observation has been confirmed by the more extensive observations of Nebel and Ruttle (1936). Goodspeed, Uber, and Avery (1935) consider the somatic anaphase chromosomes of *Lilium longiflorum* as constituted of two parallel chromatids each having two chromonemata presenting an intertwined aspect, though he is not certain about their real inter-relationship. He agrees with Nebel (1933) in the view that four free parallel chromonemata are present in each telophase chromosome. Two interlaced chromonemata in each chromatid at anaphase could not become completely free by telophase even by a new spiralization in each strand. The chromosomes are seen to increase in length after anaphase, and if a new spiralization takes place at anaphase it would only shorten the chromosome length. The other method by which they could become free is by the untwisting of each chromonema. The spatial limitation of the telophase nucleus would prevent such a process being carried to an appreciable extent and moreover such untwisting is not observed at that stage. It is thus seen that the daughter chromonemata could not have been intertwined or interlocked and they were more probably free from their inception.

It was seen that the early anaphase chromosomes have two free spiral chromatids approximated to the borders of the chromosomes and in some

cases these two chromatids were twisted on each other at two or three regions. In the late anaphase chromosomes an increase was observed in the number of twists between chromatids. At early prophase when the two chromatids were released from their close association it was seen that the number of twists between chromatids were still larger. There could be no doubt that this has been brought about by the changes undergone by the chromosomes subsequent to anaphase separation. Sharp (1929), Hedayetullah (1931), and Perry (1932), who have all figured free parallel chromatids at anaphase, have shown interlaced chromatids at prophase. Kaufmann's (1926) figures of the somatic anaphase chromosomes of *Podophyllum peltatum* show a maximum of three twists in one chromosome, whereas his figures of prophase show up to seven or eight twists in a few chromosomes (see p. 359; figs, 19-23). An examination of the figures given by Koshy (1933) and Hoare (1934) also reveal the same fact. Hedayetullah (1931) has stated that chromatid split and twisting of the split halves are nearly simultaneous processes and take place at metaphase. But he has described an untwisting of chromatids at telophase and leaves unaccounted for the twisted aspect of chromatids at prophase, which he as well as several others have observed. Darlington (1936), who has denied the duality of anaphase and telophase chromosomes, states that 'chromatid coiling at metaphase seems to be developed during prophase chiefly as a result of a strain imposed on the chromatids by spiralization, but subject also to other conditions that have not yet been ascertained'.

The twisting of the two free spiral chromatids on each other is comparable to the twisting of homologous chromosomes subsequent to pairing at the prophase of meiosis. Differential contraction might be responsible for the increase in twisting up to telophase. The prophase chromatids are more twisted on each other and the twists are mainly associated with the persisting telophase spirals, and so it appears possible that differential expansion also contributes to an increase in twisting between chromatids. Even after the close association of the two chromatids at mid-telophase chromosome elongation continues. If in the process of elongation the ends of chromosomes also rotate in opposite directions to a certain extent, there will be proportionate twisting between the two chromatids. This seems more likely as the increase in size of the nucleus prior to interphase does not keep pace with the elongation of the chromosomes. This satisfactorily accounts for the presence of chromatid twists at some of the gyres of the persisting telophase spirals and the twists seen at other regions are probably the twists brought about prior to telophase and subsequent to anaphase separation.

From early prophase the nucleus enlarges in size up to the dissolution of the nuclear membrane at late prophase. With this increase in volume of the nucleus and the shortening of the chromosomes due to spiralization, the chromosomes are more spaced out and also appear peripherally arranged by mid-prophase. With these changes is associated a straightening of the chromosome as a whole. This straightening also removes most of the persisting

telophase spirals and as the twists are also seen reduced, it is presumed that this straightening involved a slight rotation of the chromosome also. This rotation with contraction, since it reduces the number of twists, must have been in the opposite direction to the rotation with elongation at telophase. Thus it appears that a reversal of the telophase changes, which led to an increase in the number of twists, removes nearly all the twists by metaphase.

It is seen in somatic mitosis that the minor spiral seen at early prophase appears as the major spiral at the prophase of the succeeding division. Thus the chromosome visibly remains as a spiral throughout the nuclear cycle in somatic mitosis and for a short period at prophase it is seen as a double-coiled spiral. At late metaphase two free parallel spiral chromatids are seen in each daughter chromosome. Due to the subsequent twisting of the chromatids, at all other stages in the somatic mitosis we get a 'spiral-twisted-on-spiral' structure (cf. Koshy, 1934, p. 112).

The next point for consideration is how the minor spiral originates and whether it is the 'ultimate spiral' or not. Physico-chemical investigations have shown that the molecules of some crystalline substances exist in the form of chains. Bernal and Crowfoot (1934), who made X-ray studies of the protein pepsin, have observed a 'molecular spiral' during contraction. They take the molecular chain as probably formed by a degeneration resulting from a linking up of amino-acid residues. They further suggest the probability of the molecules of the primary soluble proteins having their constituent parts grouped more symmetrically around a prosthetic nucleus. Astbury and Lomax (1934) are inclined to consider the initial unit as the molecular chain itself and the spiral arrangement of the pepsin molecules as a subsequent process which may only be an elaboration of the intra-molecular folding which Astbury (1933) observed in keratin transformation. It emerges from the above findings that the visible patterns are determined by the arrangement of the molecules themselves.

From evidence discussed earlier one is led to the conclusion that the mechanism of spiralization is associated with a compensating internal twist. Since the visible minor spiral is shown to depend on the ultimate molecular arrangement, the reversed internal twist should have originated in the latter itself. It may be that this twist is determined by the relative arrangement of adjacent molecules in a spiral with reversed twists with reference to a prosthetic nucleus (Bernal and Crowfoot, 1934) or a central axis. Darlington refers to this twist as the 'molecular spiral'. Koshy (1933), basing his view on Earl's (1927) conception of the gene-thread as the basis of chromonema, has stated that it is highly probable that the particles which compose the chromonema are arranged spirally along its axis.

It was seen that the major spiral results from the development of the minor spiral. The first visible spiral is the minor spiral and it may be the molecular spiral increased in dimensions as a result of growth. If the ultimate arrangement of molecules remains the same at all stages, then this growth would

involve the formation of a new molecular spiral. So the new spiralization seen at the prophase of a nuclear division is in all probability determined by the organization of an invisible molecular spiral, which will become the minor spiral of the immediately succeeding division, and this in its turn becomes the major or relic spiral of the next division.

With the acceptance of the chromosome theory of heredity together with the conception of the chromonema as the permanent constituent of the chromosome it has to be conceded that the gene-string is located in the chromonema. Calculations of the average size of a gene give values from 20 to 70 m. An object of this size cannot normally come within the range of visibility with the optical instruments which we now use. The regularity in behaviour of the chromosome suggests that the genes are definitely organized self-reproductive units. This leads us to assume that they are of a higher order of complexity than single molecules. It is probable that definite portions of the molecular spiral represent particular genes. This conception of the molecular spiral tends to support Morgan's (1928) theory of the linear arrangement of the genes.

(c) *Time and mode of chromonema and chromosome division.*

Most of the earlier workers on somatic mitosis considered the chromosomes as homogeneous structures splitting at the prophase of the nuclear division in which chromosomes so formed separated. A very large number of recent investigators have shown the origin of split in each chromosome as occurring at or before the metaphase of the preceding division. But the time and mode of cleavage have been variously interpreted even by investigators who agree regarding the duality of structure. Kaufmann (1926), Sharp (1929), Telezynsky (1931), Tuan (1931), and Smith (1932) obtained evidence to show that the chromonema division takes place in the late prophase leading to a quadruple structure from that stage up to anaphase separation. Koshy (1933, 1937), Hoare (1934), Naithani (1937), and Atwood (1937) have shown that the chromonema division is a pro-metaphase process. Hedayetullah (1931) and Perry (1932) observed quadruple structure only at metaphase and hence assumed that chromonema split and chromosome division occur simultaneously at metaphase.

In *Lilium* it is found that each somatic chromosome consists of two chromonemata and the division of each chromonema is seen to precede the separation of each chromatid at metaphase. At mid-prophase duality is observed at certain regions and it becomes particularly clear at late prophase. Since the daughter chromonemata are associated very closely in a common spiral, the duality could not be made out along the whole length of the chromatid. At metaphase the chromonemata in each chromatid are seen distinctly separate and though there is no common matrix binding the chromatids, each chromatid possesses its individual matrix. In preparations in which the spiral structure is obscure chromatid duality is observed only at metaphase when the

two chromonemata separate to the borders of the chromatid matrix. Hedayetullah and several others failed to observe the spiral structure of the chromosomes at this stage and it is no matter for surprise that they did not obtain any evidence of chromatid duality which would have been present in these spirals. Hsu Siang (1932) observed in the early prophase chromatids in *Lilium tigrinum* the individual chromatids having an irregular outline, 'strongly suggesting that they are each composed of two tightly twisted threads'. He observed a quadruple structure only in a few chromosomes, and that too only at metaphase. The irregular outline to which he refers only shows the presence of the internal spiral which he failed to observe.

It is to be expected, as stated earlier, that gene division would precede chromonema division. While gene-reproduction may be a rapid process, the synthesis and accumulation of chromatin and other by-products of its activity may be comparatively slower processes. The duality in the chromatid becomes visible only after the two daughter gene-strings have increased in size and separated sufficiently to allow them to be optically distinguishable. It is therefore not unlikely that cleavage might have originated at early prophase at least. Thus each chromosome may be considered as quadruple from early prophase. It is also probable that the onset of a normal nuclear division marks the beginning of reproductive activity in the genes and the initiation of processes leading to chromonema duplication may be considered as the normal cause of the transformation of the nucleus from the 'resting' to the 'kinetic' condition. Kaufmann (1936, p. 536) states that chromatid doubleness 'and the consequent quadripartite nature of each chromosome may not be evident until late prophase or early metaphase, although actual division apparently occurs much earlier, the split being obscured by the close approximation in pairs of the half-chromatids'.

Kuwada (1926), from a study of the structure of the somatic chromosomes of *Vicia Faba*, found that the anaphase and telophase chromosomes are constituted of single spiral threads which at the succeeding prophase become straight prior to longitudinal splitting. Later, Kuwada and Nakamura (1935) as a result of investigations in living staminate hair-cells of *Tradescantia*, have concluded that 'two chromonemata are contained in each chromosome in the anaphase and telophase but they are interlaced with each other and cannot be separated'. Kuwada (1933) was the first to suggest a mechanism of spiralization which would account for the separation of daughter spirals without entangling. Huskins and Smith (1935) have supported this mechanism of spiralization. Nebel (1933) has suggested that if reduplication of chromonema occurs in only one plane, passing through the main axis of the chromosome, no difficulty will arise in the separation of the daughter chromonemata. Darlington (1935) is inclined to consider that the mechanism of spiralization is determined by a twist in the ultimate chromonema spiral. Evidence from the present study, though contradictory to several of Darlington's views on chromosome structure, has shown it as highly probable that the visible new

spiral at the prophase is determined by a reversed twist in the molecular spiral. The observation of interlaced chromonemata at anaphase and telophase led Kuwada and Nakamura (1935) to assume that cleavage in a spiral would give rise to two interlaced spirals as shown earlier by Koshy (1934). This would mean that Kuwada has abandoned his earlier explanation of the mode of spiralization, namely, that for each turn of the spiral there is a *twist of the two threads* about each other in the opposite direction, so that the two coiled threads may separate, without entangling or uncoiling, as occurs in the dyad of *Tradescantia* at late first metaphase in meiosis (see Kaufmann, 1936, pp. 542-5). Whatever may be the modes of spiralization or cleavage, in *Lilium* it is found that the interlaced aspect seen at late anaphase and telophase is not the result of cleavage in a spiral chromatid, as the early anaphase chromosomes have two free parallel spiral chromonemata and the interlaced aspect is seen only later.

Kuwada and Nakamura (1935) are inclined to the view that chromonema division takes place at the interphase of the division preceding the one in which the halves so formed separate. They have stated that the chromonemata are most shrunken at this stage and division could take place only then. No convincing evidence is put forward to show that chromonema division could take place only when it is most shrunken. Further, the observations on fixed materials tend to show that the chromosomes are apparently most relaxed at the interphase (Koshy, 1937).

Brief reference may here be made to the interpretations of some recent authors which widely differ from evidence obtained in the present study. Darlington (1935, 1938) is alone among recent investigators in holding the view that the anaphase and telophase chromosomes are structurally single. Critical cytological investigations on the structure of chromosomes beginning with the work of Kaufmann (1926) have convincingly shown that each somatic metaphase chromosome is four-partite and consequently duality a constant feature in chromosome make up. Darlington (1926) described a quadruple structure of the metaphase chromosomes in the pollen grains of *Hyacinthus* and *Scilla*, but later discounted such observations as optical illusions due to the 'hollow' nature of the chromosomes. After the lapse of more than a decade during which period critical experimental and observational evidence have accumulated in support of duality of chromosomes, Darlington (1938) has changed his ground and considers all cytological observations of anaphase duality as based on misinterpretations of the 'bubbles of differential refractivity' arising in the chromosome as a result of using fixatives with acetic acid. Though a variety of fixatives were tried in the present study, nothing that could be interpreted as 'bubbles' in the chromosomes or chromatids was seen in any preparation. Further, Gates and Mensinkai (1938) have shown that the duality observed in the somatic anaphase chromosomes of *Trillium sessile*, and illustrated with excellent photomicrographs (Gates 1937), is not an artefact induced by fixatives containing acetic acid, as similar

structures have been observed in material treated with non-acetic fixatives. Preparations of *Lilium* showing optically homogeneous structures after deep staining show duality and spiral aspect when properly de-stained. In Pl. XIX, Fig. 22, the photomicrograph clearly shows two chromosomes in which two free and parallel spiral chromonemata are seen without any appearance even remotely resembling what is illustrated by Darlington and La Cour (1938; see Text-figs. 1 and 2, and Pl. XXIV, Fig. 15). If the intertwined aspect of the chromonemata so frequently reported in anaphase chromosomes is to be taken as due to the presences of 'bubbles', and not on account of any inherent duality, as Darlington would have it, how then are we to interpret similar intertwined appearances seen in prophase chromosomes (Koshy, 1933)? The general acceptance (cf. Gates, 1938) of the presence of a pair of coiled chromonemata in all phases of chromosome history has invalidated most of Darlington's hypotheses of chromosomes and the relation of meiosis to mitosis.

Goodspeed (1935) considers that anaphase and telophase chromosomes in *Lilium longiflorum* are composed of four chromonemata, an eight-partite structure being seen at metaphase, the division in each chromonema taking place at the interphase. Nebel (1933) and Nebel and Ruttle (1936) have shown that the telophase chromosomes in *Tradescantia reflexa* and *Trillium erectum* are each constituted of four free parallel chromonemata the division in each taking place at metaphase, two mitotic cycles prior to their separation. No evidence in support of quadruple structure at anaphase and telophase was obtained in the present investigation. In my preparations the dual strands composing each telophase chromosome are seen to approximate so closely as even to obscure its double nature and they are coiled into an apparently single stranded spiral. While fixatives may be responsible to a certain extent for the appearance seen at the various stages of the nuclear cycle it seems unlikely that variations within such wide limits would really occur.

VI. SUMMARY

The chromosome number for *Lilium neilgherrense* is determined as $n = 12$; $2n = 24$. The morphology of the chromosomes is described.

Each chromosome at early anaphase in somatic mitosis consists of two free parallel spiral chromonemata embedded in a less chromatic matrix. Subsequent to anaphase separation a progressive twisting of the two spiral chromonemata takes place, giving rise to a 'spiral twisted on spiral' structure at all later stages.

At telophase the spirals relax and the two chromonemata become so closely associated that they are seen as only single-stranded spirals.

The spiral organization of the chromonemata is maintained at interphase also.

A new, visible spiralization takes place at early prophase in each chromatid

and the first evidence of this is seen as a regular corrugation in the apparently single-stranded chromosome thread. When duality reappears at early prophase, the chromatids are seen as twisted more on each other than at the previous anaphase. This twisting is presumed to be due to inter-chromatid adjustments induced by chromosome elongation in a limited space at telophase.

Cleavage in each chromatid is initiated at early prophase and the plane of cleavage is equatorial along the spiral and is such that two free spirals originate from the parent spiral. By late prophase each chromosome becomes visibly quadruple.

Thus each separating daughter chromosome at metaphase has two free parallel spiral chromonemata.

The possible methods of spiralization are discussed and it is shown that the ultimate structure of the chromonema is in all probability a 'balanced spiral', i.e. a spiral in which the twisting caused by spiralization is compensated by a reversed internal twisting at every gyre of the spiral.

In conclusion, I wish to express my gratitude to Professor T. K. Koshy, under whose supervision this work was done, for valuable suggestions and criticisms during the course of this investigation. My grateful thanks are due to Professor R. Ruggles Gates, for the keen interest he took in the progress of this investigation and for helpful criticism in the preparation of this paper.

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EXPLANATION OF PLATES XVIII AND XIX

Illustrating Mr. Abraham's paper on 'Chromosome Structure and the Mechanics of Mitosis and Meiosis. I. Mitosis in *Lilium*'.

All figures in Plate I were drawn at table level with the aid of a Zeiss camera lucida. Leitz 1/12 in. oil immersion N.A. 1.3 and 1/16 in. oil immersion N.A. 1.32 were used with Leitz periplanatic oculars. Unless otherwise indicated, the figures are drawn from cells of young ovaries fixed in Navashin's fluid according to the method described on p. 546. Figs. 1 and 17, $\times 2,200$; Figs. 19 and 20, $\times 1,800$; Fig. 18, $\times 1,500$; all other figures have a magnification of $\times 2,650$.

The photomicrographs¹ (Figs. 22-5) were taken from permanent preparations made from material fixed according to the long schedule of Navashin fixation and stained with gentian violet (Zeiss 100 \times 15; bellows extension, 9 in.).

PLATE XVIII

Fig. 1. Early anaphase chromosomes showing two free parallel spiral chromonemata surrounded by a less chromatic matrix. In a few chromosomes, the chromonemata are twisted on each other.

Fig. 2 a and b. The chromosomes at early anaphase each showing two spiral chromonemata twisted on each other.

Fig. 2 c. Duality and twisted aspect of chromosomes from material fixed in Benda's fluid. Spiral nature of chromonema not visible. In the middle chromosome no twists are seen.

Fig. 3. 'Tassement polaire', before cell-wall and nuclear membrane are formed. Note duality and twisted aspect.

Fig. 4. Late 'tassement polaire'; duality and twisted aspect clear in projecting arms of chromosomes.

Fig. 5. Telophase nucleus soon after nuclear membrane was organized. Note duality as well as beginning of close association of the two spiral chromonemata.

Fig. 6 a-c. Early telophase chromosomes showing duality and twisted aspect as well as close association of the two chromonemata.

Fig. 7. Late telophase showing apparently single-stranded spirals. The more or less straight arrangement of the chromosomes is noticeable.

Fig. 8. Nucleus emerging from interphase. Note spiral structure of the chromosomes and discontinuous chromatic dots which show a spiral course.

Fig. 9. Early prophase. Chromosome spirals are more clearly seen but duality is not yet visible.

Fig. 10. Prophase (later than stage shown in Fig. 9), from a deeply stained preparation. Duality is visible in each chromosome at several regions. Chromosome threads appear smooth.

Fig. 11. Nearly same stage as above. Duality is conspicuous and each chromatid is organized into a minor spiral. Where duality is not visible chromosome thread appears merely corrugated. The two chromatids are twisted on each other, but at some points where the chromonemata cross each other one is superposed on the other.

Fig. 12. Nucleus near mid-prophase. Spiral structure of chromatids more clear.

Fig. 13. Mid-prophase nucleus showing the major spiral considerably relaxed and the minor spiral more conspicuous.

Fig. 14. Mid-prophase showing duality in a few chromatid spirals (see arrow-marks). The major spirals are seen mostly relaxed and the chromosomes show a straight arrangement.

¹ I am indebted to Prof. R. Gopala Iyer, Director of the Madras University Zoology Research Laboratory, for kind permission to take the photographs in his laboratory. My thanks are due to Dr. J. P. Joshua and Dr. M. K. Subramaniam for kindly assisting in taking the photographs.

Fig. 15. Late prophase after nuclear membrane and nucleolus have disappeared (from a young anther cell). Twists between chromatids reduced.

Fig. 16 *a-f*. Chromosomes from early metaphase to late metaphase. Each chromosome half has two spiral chromonemata which are completely free in some cases. Details referred to in text.

Fig. 17. Some late metaphase chromosomes showing two free spiral chromonemata in each daughter chromosome. In a few chromosomes anaphase separation has commenced.

Fig. 18. Metaphase plate from the second post-meiotic mitosis at the chalazal end in embryo-sac, showing the triploid complement of thirty-six chromosomes—slightly spaced out in drawing. Three each of the S₁, S₂, and J chromosomes could be easily made out.

Fig. 19. Metaphase chromosomes of second post-meiotic mitosis at the micropylar end in embryo-sac showing the haploid complement of twelve chromosomes, drawn seriatum to show the morphology of the chromosomes (denoted by the letters S₁, S₂, J, and A-I).

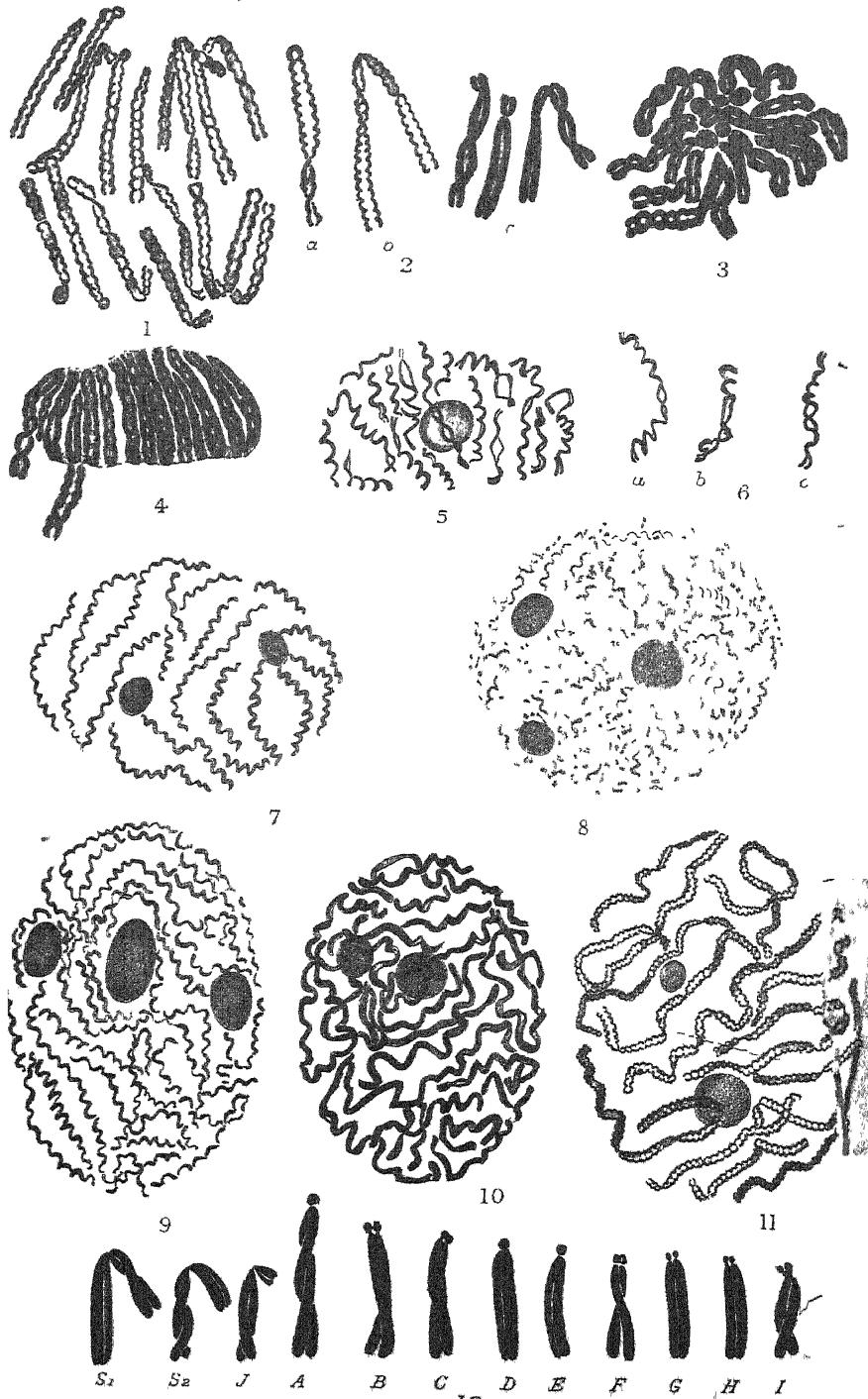
PLATE XIX

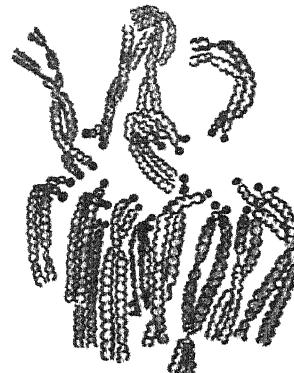
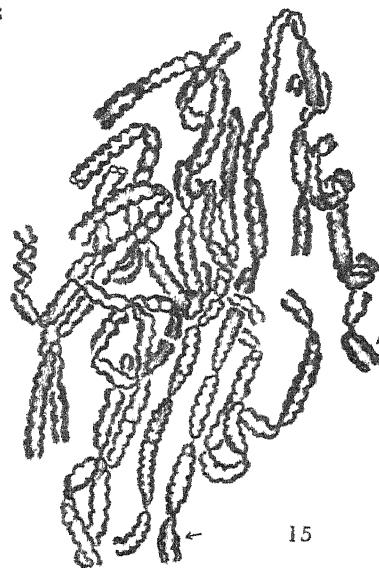
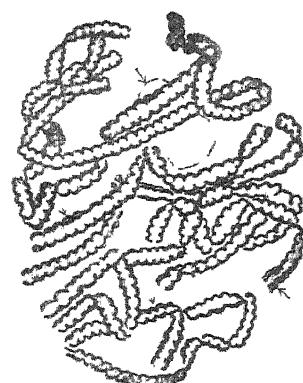
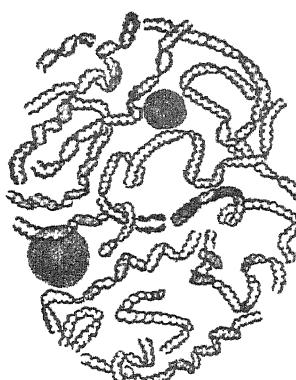
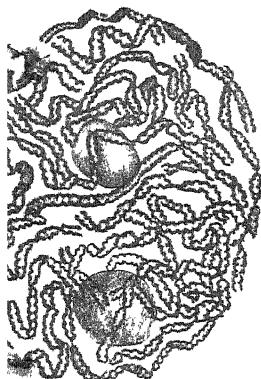
Fig. 20. Metaphase plate from a root-tip cell (fixed in Benda's fluid). Note the morphology of the twenty-four chromosomes.

Fig. 21 A and B. Photographs of spiral models made from indiarubber pressure-tubing of narrow bore, painted white on one side. A tight fitting pliable copper wire is inserted into the tube to keep it in position. Fig. 21A shows the 'Unbalanced Spiral'; the longitudinal halves intertwine with each other at every gyre of the spiral. Fig. 21B shows the 'Balanced Spiral', with reversed internal twisting at every gyre of the spiral. The longitudinal halves could separate without entangling.

Figs. 22-4. (Photomicrographs of anaphase chromosomes). In Fig. 22 the spiral nature of the two free and parallel chromonemata in each chromosome could be seen. Only two chromosomes in the upper group are in focus; the others also reveal the same structure, some of the chromosomes in this group are drawn in Fig. 1. In Figs. 23 and 24 several anaphase chromosomes show duality.

Fig. 25. Metaphase chromosomes (slightly faded) showing duality in each daughter chromosome. Note chromosome pointing to 7 o'clock and the chromosome in the centre.



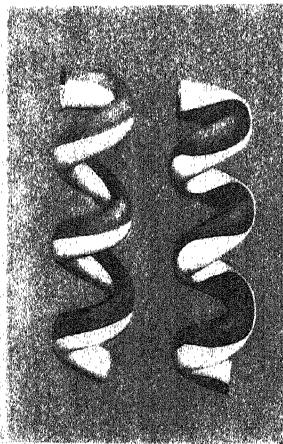




20

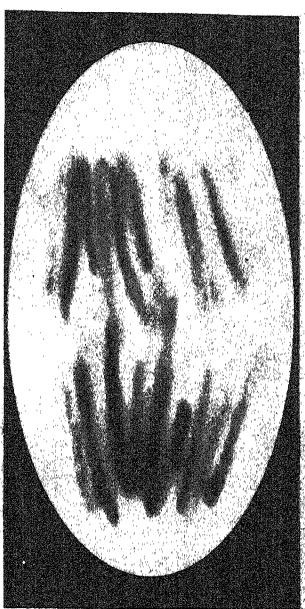
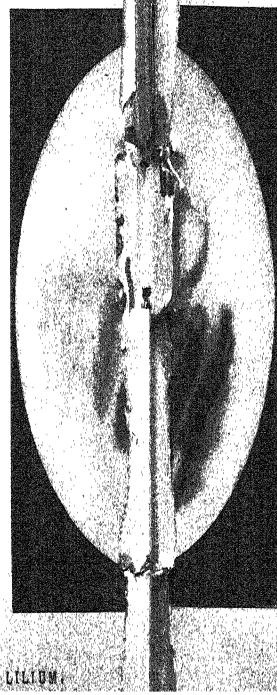


23



A B

21



24

Bud cell

Studies on the Partition of the Mineral Elements in the Cotton Plant

I. Preliminary Observations on Nitrogen and Phosphorus¹

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With seven Figures in the Text

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I. INTRODUCTION

STILES (1936) remarks that 'not all the elements absorbed are necessary for the plant, and even in the case of essential elements it does not follow that the whole amount absorbed is actually utilized'. It seems clear that the absorption of even an essential element is not dependent on requirement. Thus, as the supply of an essential element is gradually increased, sooner or later a stage is reached at which further increments in the supply are without effect on the production of dry matter, even though uptake of the element continues. On the other hand, as we shall show in the present paper, the utilization of nitrogen and of phosphorus appears to be in a large measure conditioned by requirement.

That both essential and non-essential elements are absorbed by the root and that a varying proportion of the former may play no part in furthering growth (increase in dry weight) is now usually recognized. The fraction which plays

¹ Paper No. 19 from the Physiological Department of the Cotton Research Station, Trinidad.
[Annals of Botany, N.S. Vol. III, No. 11, July 1939.]

no part in furthering growth is sometimes said to be 'luxury consumed'. In the case of an element that is not essential, and which is apparently without effect in promoting the production of dry matter (e.g. chlorine), the whole amount absorbed is presumably luxury consumed. It would appear that some elements are utilized in furthering growth without chemical transformation (e.g. potassium), and there are indications that other elements may be chemically transformed (e.g. nitrogen into amide nitrogen (Maskell and Mason, 1929)) without playing any part in the metabolic processes that affect growth. There is also the possibility that an element may be utilized in *differentiation* and take no part in growth (increase in dry weight).

Gericke (1930) attempted to distinguish between the utilized and non-utilized fractions. He planned 'to distinguish between the total quantity of an element that may be contained by rice plants and that quantity which is, in a *specific and unique way*,¹ cause for growth'. The method adopted by Gericke consisted in feeding a full nutrient solution to the roots for varying periods and then omitting one element at a time. From the growth made he hoped to assess how much of the element caused growth and how much was absorbed as a result of growth. One objection to the method lies in the fact that the omission of one element from a complete solution may affect the uptake of other elements, which may in turn affect growth. This, as Gericke himself suggested, may be the explanation of the increased growth caused by the omission of certain elements. Gericke apparently assumed that as long as an element is limiting or controlling growth, it is completely utilized, so that luxury consumption only begins when an element is absorbed without any increase in dry weight. It seems to the writers that this would only be true if growth were limited by one factor and not, as appears to be the case, controlled by a number of factors.

Thomas (1933) too thought that 'Theoretically a distinction must be drawn between the amount of an element absorbed and the amount utilized by the plant'. He pointed out that if the luxury fraction² is returned to the soil via the roots before maturity, analysis of the plant at maturity will give the utilized fraction. He found that some of his results did not conform to this concept. He says: 'Some authorities claim that the absorbed but unutilized fraction of a nutrient element is unchanged and is found as dissociated inorganic salts in the vessels and cell-sap.' As he remarks, 'Critical experiments on the subject have yet to be conducted'. It will be observed that there are two claims: (1) that the luxury fraction is chemically unchanged, and (2) that it is located in the cell-sap (vacuole).

In this preliminary survey we have separated only the nitrogen and phosphorus of the leaf into two fractions, one that is in solution in the sap and

¹ Italics by present authors. The assumption is that there are chemical forms which are especially associated with growth and metabolism.

² As will be pointed out in the discussion it appears probable that much of the luxury fraction migrates from the vegetative to the reproductive parts of the plants. There will admittedly be losses from leaves during rain and from roots during senescence.

another that is not sap-soluble. In one experiment the sap-soluble fractions have been further subdivided by determining nitrates and phosphates. No attempt has been made in this paper to distinguish between the nitrogen and phosphorus present in the vacuole and in the protoplasm (Phillis and Mason, 1937).

II. METHODS

Three experiments are described in the present paper. In the first the supply of nitrogen, in the second the supply of phosphorus, and in the third the supply of potassium was varied from a condition of starvation to one of excess. The experiments all conformed to a similar plan. The supply to the roots was varied and the effect observed on the dry weight and on the amounts of soluble and insoluble nitrogen and phosphorus in the *leaf* (lamina). The plants were collected for analysis when they were approximately 9 weeks old, that is to say, while they were still in the vegetative state. The total amount of potassium in the leaf was also ascertained in each experiment. For the whole plant the total uptakes of nitrogen, of phosphorus, and of potassium were determined in two experiments and are referred to in the discussion. The same basal culture solution was used throughout and the plants were grown in sand-culture under glass.

The results are expressed on the sample basis. The methods of sap expression and of calculating weights of the soluble and insoluble fractions have already been described (Mason and Phillis, 1936). We have expressed the *insoluble* fractions of the two elements, nitrogen and phosphorus, as percentages of the total amounts of these elements present in the leaf. The values thus obtained have been termed the *partition indices*. Crystallloid nitrogen has been used in place of total sap-soluble nitrogen. Soluble phosphorus includes all the phosphorus in the sap. In one experiment nitrate nitrogen and phosphate phosphorus in the sap were also determined. Nitrate was determined as ammonia after reduction with iron and phosphate on charcoal cleared sap (Mason and Phillis, 1936) as ammonium phosphomolybdate.

III. VARIABLE NITROGEN SUPPLY (EXPERIMENT I)

A. Procedure.

Sea Island cotton plants were grown in sand-cultures in two glass houses which will be referred to as houses A and B respectively. In each house there were 5 groups of 30 plants and each group had a different level of nitrogen supply (see Fig. 1). Nitrogen was supplied as ammonium nitrate. About the time when flowering began representative samples of 20 plants were drawn from each group in each house. Only leaves with fully expanded laminae were used for the determination of the *partition index*. The nitrogen content of the seeds of plants that completed the fruiting cycle was determined in this experiment and is referred to in the discussion.

B. Results.

The changes in the dry weight and in the weights of nitrogen, phosphorus, and potassium in the leaf are shown in Fig. 1. The weights are expressed as

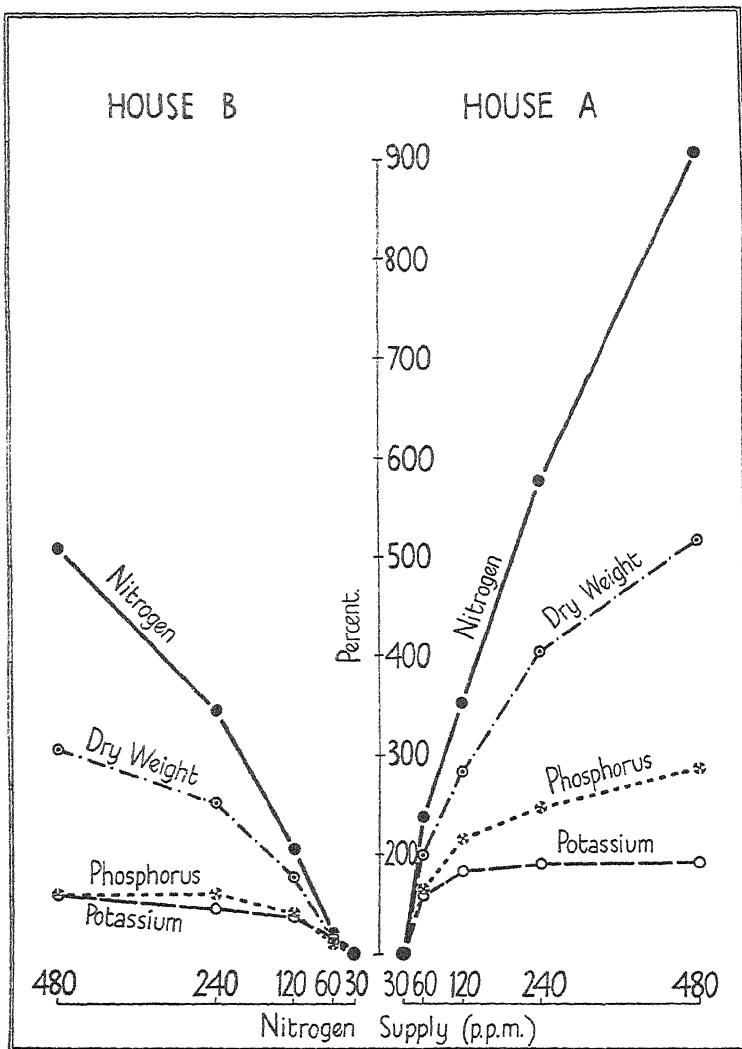


FIG. 1. Changes in the dry weight and in the weights of nitrogen, phosphorus, and potassium in the leaf under varying nitrogen supply. Results expressed as percentages of the initial values. (Experiment 1.)

percentages of the values at the lowest supply (i.e. initial values). The results for the whole plant are not shown as they agree very closely with those for the leaf (cf. Table I). It will be seen that as the supply of nitrogen increased the

dry weight increments gradually declined. In both the whole plant and the leaf there were only small increases in dry weight after the 240 p.p.m. level of nitrogen supply, the increase in the whole plant being smaller than that in the leaf. In both, however, the increase in the nitrogen uptake continued almost unchecked, so that in terms of dry weight the tissues became progressively richer in nitrogen. It is not clear what factor or factors assumed control of the dry weight as the nitrogen supply was increased, but the supply of potassium may have been involved, for there was little or no increase in the uptake of this element after the 120 p.p.m. level of nitrogen supply. It will be noticed that the increase in the uptake of phosphorus was much smaller than the increase in dry weight production. Thus the leaf became progressively poorer in phosphorus.

The changes in the *partition indices* for nitrogen (i.e. protein nitrogen as a percentage of total nitrogen) in the two houses are shown on the left of Fig. 2. For comparison the weights of total, protein and crystalloid nitrogen per 100 gm. dry weight, expressed as percentages of the initial values, are also shown. It will be seen that from about 88 to a little more than 93 per cent. of the nitrogen of the leaf was present in the form of protein. In the two houses the changes in the *partition indices* were not exactly similar. In both, however, the *partition index* tended to vary inversely with the weight of total nitrogen per 100 gm. dry weight. That is to say, the more highly the tissues were charged with nitrogen the lower the proportion of protein nitrogen. The correlation between the *partition index* and the weight of total nitrogen per 100 gm. dry weight is, however, clearly not linear, for the changes in the *partition index* are small at low levels of nitrogen saturation and large at higher levels. The actual correlation coefficients were -0.90 in house A and -0.95 in house B. Both coefficients are fully significant ($P < 0.05$).

While in both houses the *partition indices* tended to vary inversely with the weights of nitrogen per 100 gm. dry weight, the changes in the *partition indices* at low nitrogen levels were smaller than would be expected from the changes in the weight of nitrogen per 100 gm. dry weight. This may indicate that under conditions of nitrogen starvation all or nearly all the nitrogen, both protein and crystalloid, was utilized in furthering growth, so that changes in the *partition index* were relatively small, and that as other factors assumed control of growth, the tissues became progressively saturated with nitrogen with the result that an increasing proportion accumulated as crystalloid nitrogen which played no part in furthering growth (i.e. as luxury nitrogen). This interpretation of the results assumes that all the protein nitrogen and only a part of the crystalloid are metabolically active in furthering dry weight production, and that when the tissues become saturated with these forms of nitrogen, the excess accumulates as crystalloid nitrogen, which may differ from the metabolically active forms (chemical isolation) or accumulate in the vacuole (regional isolation). It also assumes that there is *luxury consumption of nitrogen even while it is the factor chiefly limiting or controlling growth*. Actually

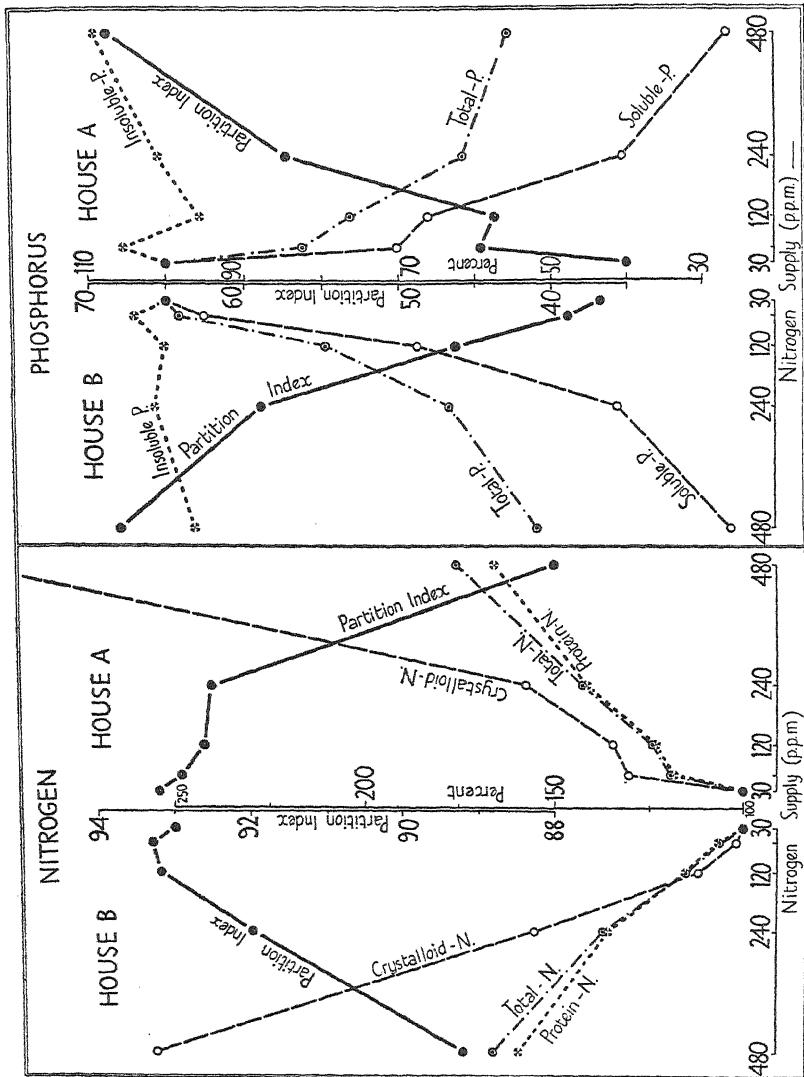


FIG. 2. Changes under varying nitrogen supply in the partition indices for nitrogen (left) and for phosphorus (right). The total weights of these elements as well as the weights of the soluble and insoluble fractions per 100 gm. dry weight are shown as percentages of the initial values. (Experiment I.)

it does not follow from the changes in the *partition index* that there is not also luxury consumption of protein nitrogen. It should be noted that there was a well-marked increase in the weight of protein nitrogen per 100 gm. dry weight. What this increase means cannot be determined until something is known about the types of protein, and the changes in the protein content of protoplasm and in the ratio of protoplasm to dry weight.

The results for phosphorus are shown on the right of Fig. 2. The *partition index* increased from about 35 to nearly 70. As it increased the weight of phosphorus per 100 gm. dry weight decreased. The correlation coefficients between the *partition index* and the weights of total phosphorus per 100 gm. dry weight were -0.95 for house A and -0.99 for house B. The correlation, unlike that for nitrogen, appears to be nearly linear. As, however, there is no evidence of phosphorus starvation, no significance can be attached to this. In fact as the weight of insoluble phosphorus per 100 gm. dry weight remained relatively constant, it might be assumed that phosphorus did not become limiting. Whether soluble phosphorus plays any important part in metabolism cannot be gauged from the results of this experiment. In comparing the behaviour of nitrogen and phosphorus it must be borne in mind (see next experiment) that the bulk of the crystalloid nitrogen is probably organic and that the bulk of the sap-soluble phosphorus is present in the form of phosphate.

IV. VARIABLE PHOSPHORUS SUPPLY (EXPERIMENT 2)

A. Procedure.

The procedure in this experiment was similar to that of Experiment 1 except that the supply of phosphorus in place of nitrogen was varied. Phosphorus was supplied as sodium phosphate. The plants were again grown in sand-culture in two glass houses.

B. Results.

The changes in dry weight and in the weights of nitrogen, phosphorus, and potassium in the leaf, again expressed as percentages of the initial values, are shown in Fig. 3. It will be seen that there was the usual decline in dry weight increments as the phosphorus supply to the roots was increased. This decline became especially marked after the 40 p.p.m. level of phosphorus supply. It should be noted that the scale for phosphorus in Fig. 3 is reduced to $1/10$ th of the scale for dry weight and the weights for nitrogen and potassium. Thus in terms of dry weight the tissues of the leaf became progressively more saturated with phosphorus as the supply of this element to the roots was increased. Both nitrogen and potassium increased less rapidly than the dry weight, so that the tissues grew poorer in these elements. It should be observed that while in the last experiment the weights of nitrogen and potassium per 100 gm. dry weight varied in reverse directions, in this experiment they varied in the same direction.

In Fig. 4 are shown the *partition indices* for nitrogen (left) and phosphorus

(right). For comparison the weights of total nitrogen and phosphorus per 100 gm. dry weight, again expressed as percentages of the initial values, are also shown. In addition to the weights of total nitrogen and phosphorus, the

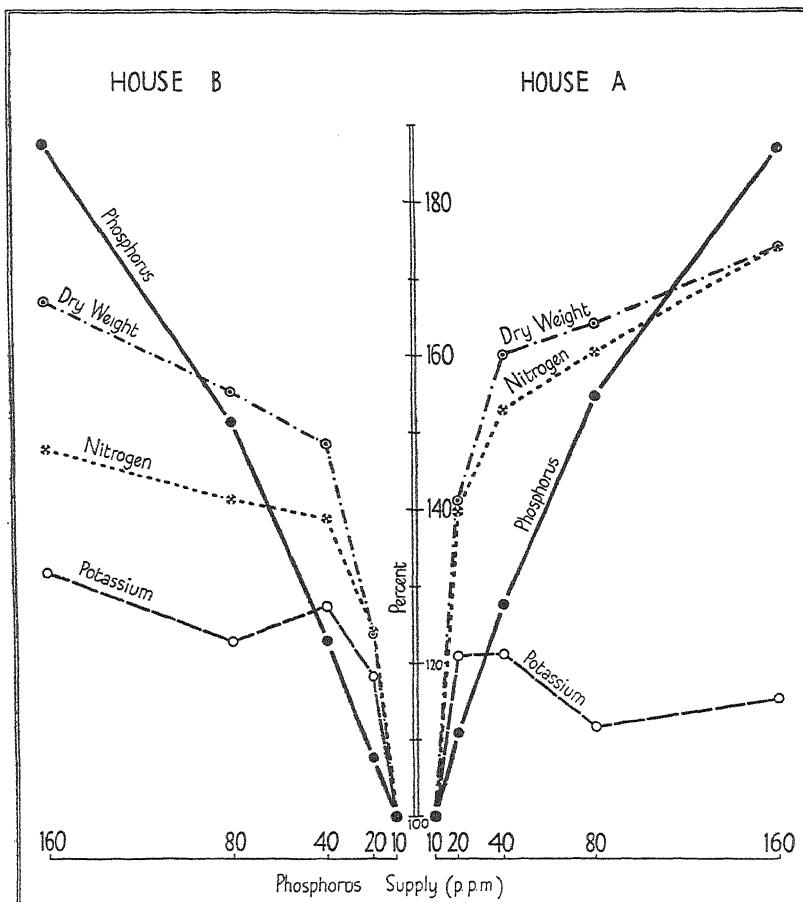


FIG. 3. Changes in the dry weight and in the weights of phosphorus, nitrogen, and potassium in the leaf under varying phosphorus supply. Results expressed as percentages of the initial values. The scale for phosphorus is $1/10$ th of that for the dry weight and the weights of nitrogen and potassium. (Experiment 2.)

weights of the soluble and insoluble fractions per 100 gm. dry weight are also recorded. For nitrogen the *partition indices* for both houses increased and became nearly constant as the supply of phosphorus was increased. The changes were greater in house A than in house B. The weights of nitrogen per 100 gm. dry weight behaved in almost exactly the reverse way. The correlation coefficients between the *partition index* and the weight of total nitrogen per 100 gm. dry weight were -0.93 for house A and -0.95 for house B. Both

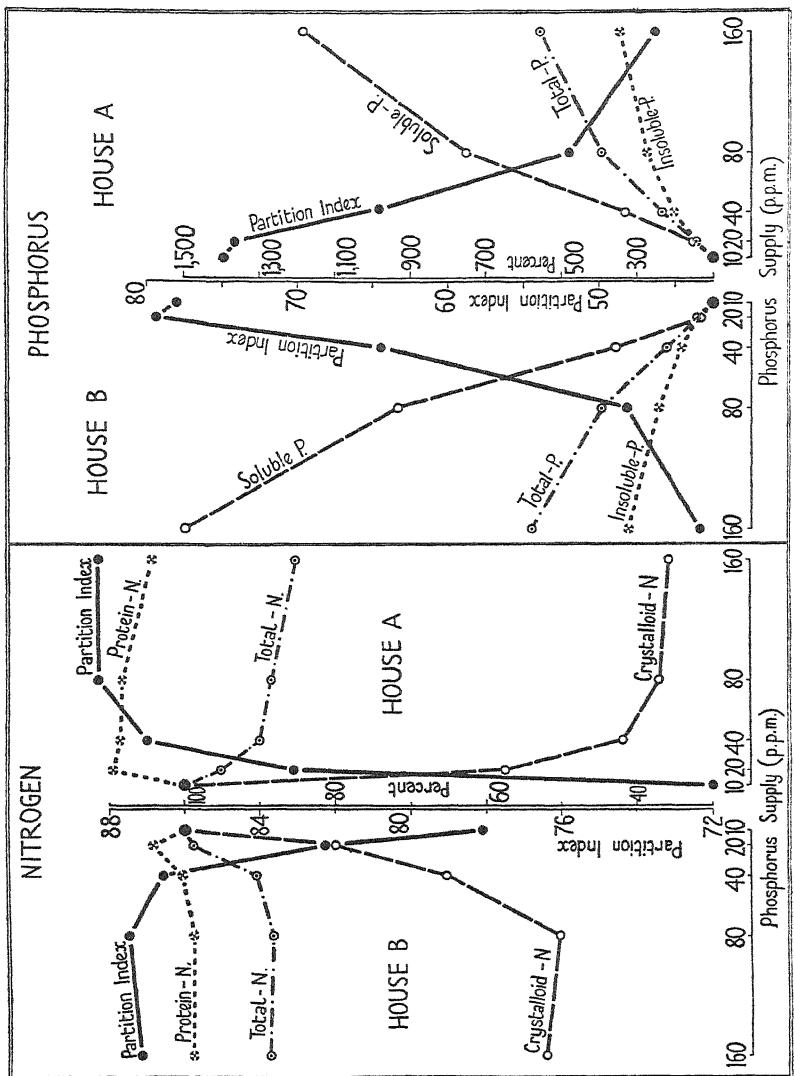


FIG. 4. Changes under varying phosphorus supply in the partition indices for nitrogen (left) and for phosphorus (right). The total weights of these elements as well as the weights of the soluble and insoluble fractions per 100 gm. dry weight are shown as percentages of the initial values. (Experiment 2.)

coefficients are fully significant. Thus the extent to which the tissues were saturated with nitrogen again appears to be an important factor determining the distribution of nitrogen between protein and crystalloid forms.

The *partition indices* for phosphorus present certain features of interest. Between the 10 and 20 p.p.m. levels of phosphorus supply it increased slightly in house B and decreased in house A. There was, therefore, no significant change in the partition of phosphorus at very low levels of supply. In both houses there then occurred rapid declines to the 80 p.p.m. level of supply, which were followed by less rapid declines. Total phosphorus per 100 gm. dry weight increased in both houses. The correlation coefficients between the *partition index* and the weight of total phosphorus per 100 gm. dry weight were -0.98 in house A and -0.96 in house B. The small change in the *partition index* under conditions of extreme phosphorus starvation may have been due, as we suggested for nitrogen, to the complete utilization of phosphorus under these conditions. This of course assumes that some of the soluble phosphorus has a claim to be considered 'utilized'. If this interpretation is accepted it would follow that phosphate phosphorus is utilized, for even under conditions of extreme phosphorus starvation the bulk of the soluble phosphorus is present in the form of phosphate.

The results for nitrates and phosphates are shown in Fig. 5. They are shown as percentages of the crystalloid nitrogen and of sap-soluble phosphorus respectively. It will be seen that while nearly all the sap-soluble phosphorus was present as phosphate, the bulk of the crystalloid nitrogen was present as organic nitrogen. The percentage of phosphate increased as the supply of phosphorus increased, but even at very low levels of supply, however, the bulk of the sap-soluble phosphorus was present as phosphates. It will also be observed that as the tissues became richer in phosphorus, phosphates were responsible for an increasing proportion of the soluble phosphorus. The same is generally true of nitrates; as the tissues became more saturated with nitrogen, nitrates were responsible for an increasing proportion of the crystalloid nitrogen. To sum up the results for nitrogen and phosphorus: not only were the soluble fractions present in larger proportions, but the inorganic forms were also present in larger proportions under conditions of plenty than under conditions of starvation.

V. VARIABLE POTASSIUM SUPPLY (EXPERIMENT 3)

A. Procedure.

In this experiment the procedure followed the same lines as previous experiments except that the supply of potassium was varied. Potassium was supplied half as chloride and half as sulphate.

B. Results.

The changes in dry weight and in the weights of nitrogen, phosphorus, and potassium in the leaf are shown in Fig. 6. The results are again expressed as

percentages of the initial values. The scale for potassium has been reduced to 1/20th of that used for the dry weight and the weights of nitrogen and phosphorus. The increase in potassium was greater than the increase in dry

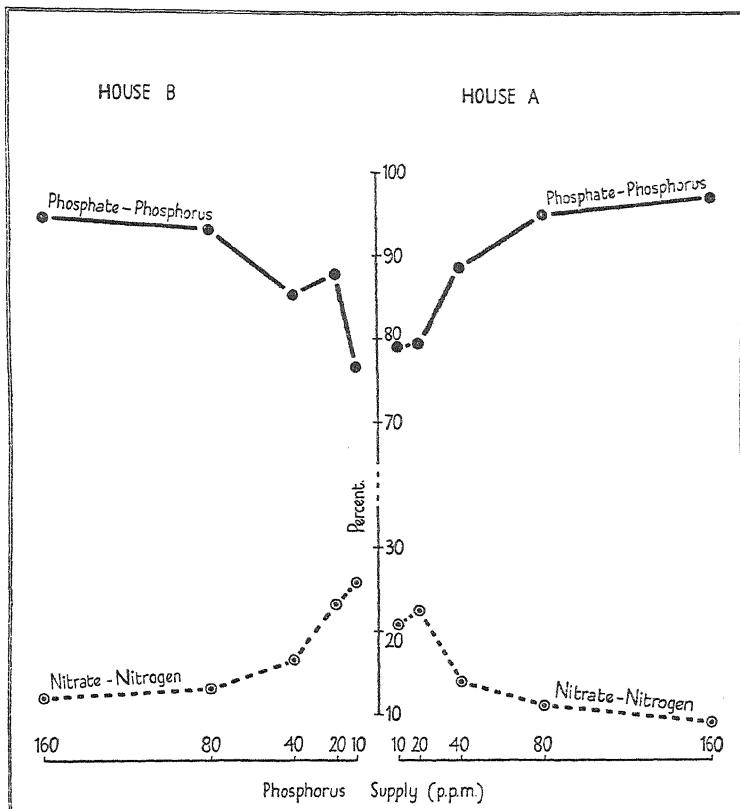


FIG. 5. Changes in phosphate phosphorus and nitrate nitrogen expressed as percentages of soluble phosphorus and crystalloid nitrogen respectively, under varying phosphorus supply. (Experiment 2.)

weight. Thus the tissues became progressively richer in potassium. The dry weight increased up to 100 p.p.m. level of potassium supply and then showed some decline, while potassium continued to increase. In the whole plant the changes in the dry weight were similar to those of the leaf except that after the 100 p.p.m. level of potassium supply instead of declining there was no further change. While the tissues of the leaf became richer in potassium (in terms of dry weight) they grew poorer in nitrogen and phosphorus. In the three experiments recorded it will have been noticed that an increase in terms of dry weight in any one of the three elements, nitrogen, phosphorus, or potassium, leads to a decline in the other two elements.

The *partition indices* for nitrogen are shown on the left of Fig. 7. For comparison the weights of total, protein, and crystalloid nitrogen per 100 gm. dry

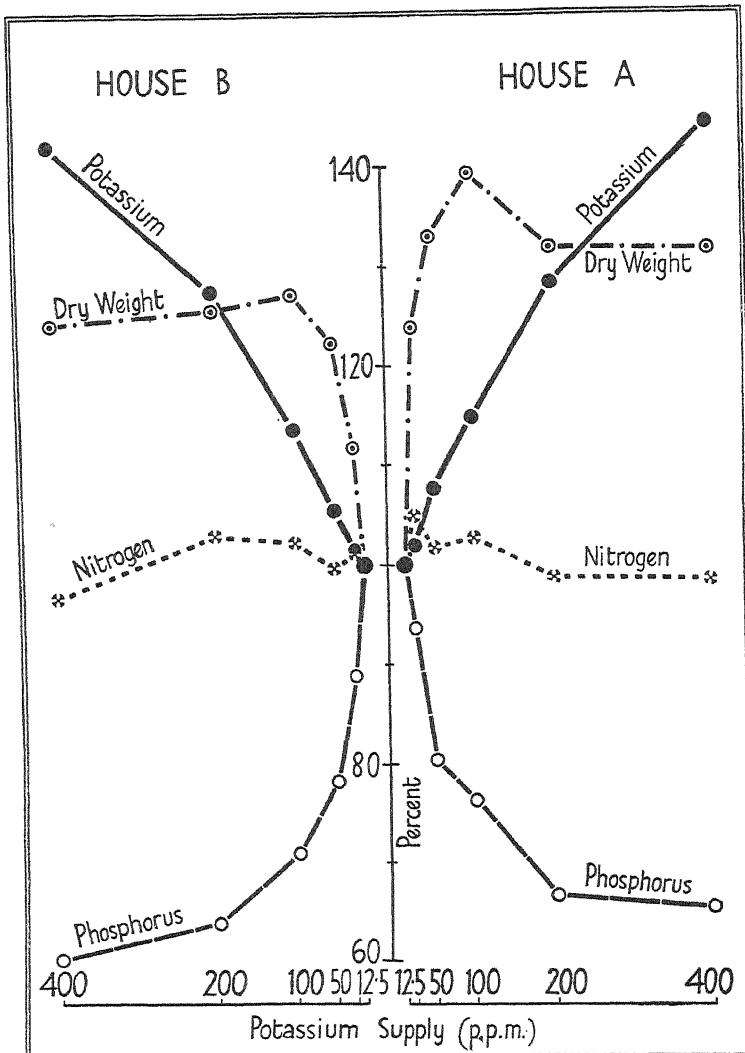


FIG. 6. Changes in the dry weight and in the weights of potassium, nitrogen, and phosphorus in the leaf under varying potassium supply. Results expressed as percentages of the initial values. The scale for potassium is $1/20$ th of that for the dry weight and the weights of nitrogen and phosphorus. (Experiment 3.)

weight are shown as in previous experiments. It will be seen that the curves for the *partition indices* in the two houses conform to essentially the same pattern as in the first two experiments. The same flattening of the curves at

low levels of nitrogen per 100 gm. dry weight will be observed, but the *partition indices*, which in this experiment reach a maximum at the same level of potassium supply as the dry weight, show, instead of a flattening of the curve, a definite decline just as the dry weight does. The correlation coefficients between the *partition indices* and total nitrogen per 100 gm. dry weight are again fully significant, —0.99 in house A and —0.93 in house B.

The *partition indices* for phosphorus are shown on the right of Fig. 7. In house B it rose from almost 52 at the 12.5 p.p.m. level of potassium supply to 62 at the 50 p.p.m. level of supply. It then declined to approximately its original value. It will be noticed that the maximum value nearly coincides with the maximum value for the nitrogen *partition index* and also, of course, for the dry weight. The results for house A differ in that an actual decline is indicated between the 12.5 p.p.m. and 25 p.p.m. levels of supply, and a small rise between the 200 p.p.m. and 400 p.p.m. levels of supply. The essential similarity of the patterns in the two houses will, however, be apparent. The maximum value in house A coincides with the maximum value for the *partition index* for nitrogen and for the maximum dry weight of the leaf.

The results for the weights of total, insoluble and soluble phosphorus per 100 gm. dry weight are presented in the usual way. It will be evident that in this experiment there are factors other than the degree of saturation of the tissues with phosphorus which determine the magnitude of the *partition index*. It would appear that at high levels of potassium supply not only is the dry weight of the leaf diminished but that the capacity of protein (protoplasm) for insoluble phosphorus is also reduced.

VI. DISCUSSION

The incompleteness of the data presented in this paper will be apparent. Until much more complete chemical and regional analyses have been carried out, it is only possible to draw tentative conclusions. Work at present in progress already shows the age of the leaf to be a factor of great importance in the partition of nitrogen and phosphorus. How far the behaviour of the leaf represents that of other tissues will also have to be elucidated. In passing, it should be stressed that the changes in the mineral uptake by the leaf may differ greatly from those of the whole plant. We show in Table I, for example, the correlation coefficients between the dry weights and the amounts of nitrogen, phosphorus, and potassium in the leaf and in the whole plant for Experiments 1 and 3, that is to say, under varying nitrogen and under varying potassium supply.

It will be seen that under varying nitrogen supply the leaf lamina is a good indicator of the nitrogen and phosphorus uptake by the whole plant, but that this is not the case under varying potassium supply. For potassium, on the other hand, the leaf is in both experiments a reliable indicator of the uptake by the whole plant.

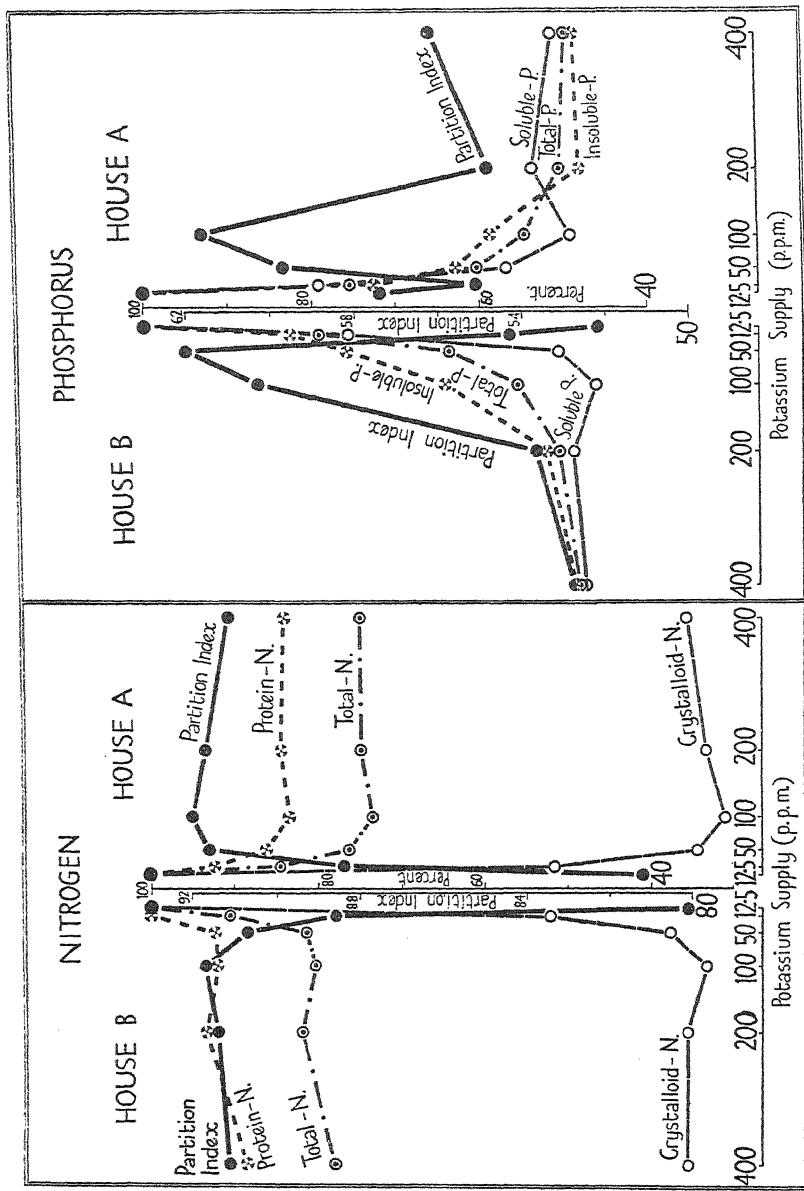


FIG. 7. Changes under varying potassium supply in the *partition indices* for nitrogen (left) and for phosphorus (right). The total weights of these elements as well as the weights of the soluble and insoluble fractions per 100 gm. dry weight are shown as percentages of the initial values. (Experiment 3.)

TABLE I

Correlation Coefficients between Weights of Dry Matter, Nitrogen, Phosphorus, and Potassium in Leaf Lamina and in Whole Plant

	Dry weight.	Nitrogen.	Phosphorus.	Potassium.
Experiment 1 (nitrogen varying)	House A +0.990	+1.000	+1.000	+0.990
	House B +0.981	+1.000	+0.997	+0.999
Experiment 3 (potassium varying)	House A +0.963	+0.267	+0.452	+1.000
	House B +0.992	+0.258	+0.662	+1.000

Fully significant values ($P < 0.05$) are italicized.

Another point that requires clarification is the relation between the storage¹ of mineral elements and their luxury consumption.² In annual plants such as Sea Island cotton the uptake of mineral elements is gradually reduced and eventually stops as the reproductive phase progresses. A large proportion of the elements absorbed during the vegetative phase is distributed to the bolls during the reproductive phase. As this proportion was used in furthering dry weight production during the vegetative phase, it cannot, therefore, be said to have been stored by the vegetative plant. We have shown in a recent paper (Mason and Phillis, 1934), however, that some nitrogen is truly stored by the tissues of the bark even though conditions of nitrogen starvation prevail. In the present paper we have suggested that there is luxury consumption of nitrogen by the leaf even while nitrogen is the factor chiefly limiting or controlling growth.

TABLE II

*Nitrogen (gm. per 100 gm. dry weight) in Vegetative Plant and in Seeds
(Experiment 1)*

	Vegetative plant.	Seeds.
30 p.p.m.	. . 1.780 (100)	2.025 (100)
60 p.p.m.	. . 2.264 (127.2)	2.801 (138.3)
120 p.p.m.	. . 2.359 (132.5)	3.301 (163.0)
240 p.p.m.	. . 2.754 (154.7)	3.996 (197.3)
480 p.p.m.	. . 3.820 (214.6)	4.484 (221.4)

In Table II we show the weights of nitrogen per 100 gm. dry weight in the whole vegetative plant before the onset of the reproductive phase, and in the seeds at the end of the reproductive phase. The data refer to Experiment 1 in which the supply of nitrogen was varied from 30 p.p.m. to 480 p.p.m. It will be seen that the range of variation is much the same in the seeds as in the vegetative plant, which suggests that the luxury nitrogen of the leaf as well as the stored nitrogen of the bark had moved into the seeds. Luxury nitrogen would therefore appear to be utilized in the seed in the same manner as stored nitrogen.

The fate of this luxury nitrogen in the seed is unknown and at present it is

¹ Absorbed without playing any part in metabolism, but subsequently utilized in some other part of the plant. ² Absorbed without playing any part in dry weight production.

impossible to say whether it is utilized in furthering the growth of the seed or not. In soils deficient in nitrogen it may subsequently assist in the production of dry matter by the seedling.

The main fact that has been brought to light in the present paper is that in the leaf the partition of nitrogen and of phosphorus between soluble and insoluble forms appears to be determined very largely by the extent to which the tissues are saturated with these elements. The insoluble fraction (i.e. for nitrogen, protein-nitrogen, for phosphorus, presumably phospho-proteins and phospho-lipoids) expressed as a percentage of the total amount of the element present in the tissues of the leaf has been termed the *partition index*. The *partition index* was found to be negatively correlated with the total amount of the element per 100 gm. dry weight. This is true for nitrogen and for phosphorus over a wide range of supply of these elements. It is also true of nitrogen, but not of phosphorus under varying potassium supply. We have suggested that the negative correlation between the *partition index* and the weight of the element per 100 gm. dry weight is due to the insoluble fraction being more intimately associated with metabolism than the sap-soluble fraction. This will probably be admitted altogether apart from the negative correlations between the *partition indices* and the saturation of the dry material with nitrogen and phosphorus respectively, for proteins and the forms of phosphorus associated with lipoids and proteins must form the main materials out of which protoplasm is synthesized.

In the case of nitrogen the correlation between the *partition index* and the weight of nitrogen per 100 gm. dry weight is not, however, linear. Under conditions of nitrogen starvation the *partition index* undergoes a much smaller change than under conditions of high saturation for a given change in nitrogen per 100 gm. dry weight. This, we suggested, was due to complete or nearly complete utilization of nitrogen, both soluble and insoluble forms, under starvation conditions. The large changes in the *partition index* under conditions of nitrogen saturation we suggested might be due to the accumulation of other forms of crystalloid (soluble) nitrogen. We showed that this luxury consumption of crystalloid nitrogen was partly due to nitrate nitrogen. If these suggestions are admitted, it would follow that the luxury consumed part of the nitrogen is present as nitrate and certain forms of organic crystalloid nitrogen. It would not of course follow that there is not also luxury (storage) protein nitrogen in the leaf.

Though the importance of the saturation of the tissue with nitrogen and with phosphorus has been stressed, it seems clear that other factors may sometimes play a dominating role in determining the *partition indices* of these elements. Thus we found that under conditions of very high potassium supply the *partition index* of phosphorus may be positively correlated with the weight of phosphorus per 100 gm. dry weight (see Fig. 7). We interpreted this to mean that when the tissues are highly charged with potassium the combining power of protein with insoluble phosphorus is diminished.

VII. SUMMARY

1. Observations, limited to the leaf, were made on the changes in the partition of nitrogen and of phosphorus between the sap-soluble fraction and the fraction that is insoluble in the sap. The insoluble fraction expressed as a percentage of the total amount of the element is termed the *partition index*.
2. Over wide ranges of nitrogen, phosphorus, and potassium supplies to the roots, the *partition index* of nitrogen was negatively correlated with the weight of nitrogen per 100 gm. dry weight.
3. Over wide ranges of nitrogen and phosphorus supplies to the roots, the *partition index* of phosphorus was also negatively correlated with the weight of phosphorus per 100 gm. dry weight, but under varying potassium supply this was not the case.
4. It was concluded that the saturation of the tissues with nitrogen and phosphorus respectively was an important factor determining the partition of these elements.
5. It was suggested (*a*) that luxury consumption of nitrogen and of phosphorus occurs even when these elements are controlling dry weight production, (*b*) that no distinction can be drawn between the luxury and storage forms of these elements.

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Epinastic Response Induced in Plants by *Bacterium solanacearum* E.F.S.

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With Plate XX and one Figure in the Text

I. INTRODUCTION

PLANTS infected by certain bacteria and viruses show stimulation effects such as epinasty or hyponasty of leaves, adventitious root formation, and gall formation. It is the purpose of this and subsequent papers to study the occurrence and physiology of these host reactions. The present paper deals with epinastic response in plants due to *Bacterium solanacearum* E.F.S. A preliminary account of certain aspects of the following research was published in *Nature* (1936 a).

II. HISTORICAL SURVEY

The epinastic curvatures of leaves of tomato as a specific reaction to infection by *B. solanacearum* were first observed by Hunger (1901) and later by Smith (1914). The latter worker stated that epinasty of leaves and the formation of adventitious roots were frequently the only external symptoms in tomato. The only other plants in which leaf epinasty has been reported are the castor-oil plant (Smith and Godfrey, 1921) and Hibiscus (Jochems and Maas, 1922).

It is of interest here to note that the epinastic response induced by *B. solanacearum* is closely similar to that induced in plants by ethylene and carbon-monoxide gases (Crocker *et al.*, 1932; Zimmerman *et al.*, 1933), and by certain chemical substances (Hitchcock, 1935).

III. METHODS

Technique of inoculation.

Plants were grown in infected soil and one or more lateral roots were broken to allow entry of the parasite, or the organism was lightly pricked into a selected bundle of the stem or root. The culture of *B. solanacearum* used was isolated from naturally diseased potato plants in Victoria.

Histological technique. Data were obtained primarily from hand-cut sections of fresh material. Further material was fixed for paraffin sectioning in 75 per cent. alcohol to which a drop or two of nitric acid had been added (Hunger, 1901). Heidenhain's stain and Rawlin's modification of Stoughton's stain

(1933) were generally used. The location of the bacteria in the vascular bundles of infected plants was determined either by sectioning or by a staining and maceration method (Grieve, 1936).

Range of plants tested. The following plants were selected for study: *Lycopersicum esculentum* Mill., *Solanum tuberosum* L., *Solanum nigrum* L., *Solanum dulcamara* L., *Ricinus communis* L., *Tagetes erecta* L., *Tropaeolum majus* L., *Impatiens balsamina* L.

Test plants were grown under cool glasshouse conditions at Melbourne University (Australia) and at the Botany School, Cambridge University.

IV. THE NATURE AND OCCURRENCE OF BACTERIALLY INDUCED EPINASTIC RESPONSE

De Vries (1872) used the term 'epinasty' to express a condition where the upper side of a dorsiventral organ grew faster than the lower. The bacterially induced movement of leaves conforms to this definition. This was demonstrated in tomato and potato plants by marking with India ink parallel lines 2 mm. apart on the upper and lower sides of the petioles near their junction with the stem. When the leaves bent down, the distances between the first three marks on the upper surface were found to be considerably increased while the distances between the marks on the lower surface were scarcely altered. Sections through reflexed petioles showed that cell elongation had occurred on the upper side (see Pl. XX, Fig. 6). Under suitable environmental conditions, leaf epinasty constantly occurred in infected tomato and potato plants, less frequently in castor-oil and African marigold plants, and not at all in black nightshade, garden nasturtium, and balsam plants. The incidence and degree of epinasty varied with the variety of tomato and potato plant used. Tomato varieties Marglobe and Burwood Wonder were found to be the most suitable as was also the potato variety Carmen.

The reacting leaves in tomato plants, grown on a side bench in a glasshouse, were mainly those in the intermediate and lower parts of the stem. The growth region was confined to the base of the petioles. In most plants, 9–12 in. in height, the uppermost two or three leaves wilted, unilaterally or bilaterally, without reflexing. Some exceptions were noted, mainly during periods of dull weather or when the light intensity was artificially reduced. Under such conditions leaves near the top reflexed and the region of growth and bending was spread over a greater length. An example of epinastic response in tomato is shown in Pl. XX, Fig. 3. Tomato plants under 5 in. in height failed to show epinasty even when grown under very humid conditions.

Measurements of angle change in typically reacting leaves on fifty infected tomato plants gave a range from 65° to 110° , with an average of 85° . The average final angle made with the stem was 135° , the average normal angle made with the stem by such leaves before reacting being 50° . In contrast to tomato, the epinastic response in potato plants was seen most distinctly and frequently in the uppermost leaves, although occurring as well in leaves lower

down the stem. Examples of epinasty in potato plants are shown in Pl. XX, Figs. 1 and 4. For leaves in intermediate positions on the stem the average angle change was 70° , the average final angle made by the upper surface of the petiole with the stem being 130° . Apical leaves, however, showed growth over the whole of their length and sometimes bent through extreme angles (see Pl. XX, Fig. 1).

The epinastic response to bacterial invasion was permanent in all cases. During summer months epinasty was followed sooner or later (one to four days, according to environmental conditions) by unilateral or occasionally bilateral wilting of the leaves (see Pl. XX, Fig. 4). A curious feature observed, particularly in well-developed plants of tomato and potato bearing several leaves, was that often a wilted leaf or one quite unaffected, would occur between two reflexing leaves.

Time relations in epinastic response.

The results of observations on the time taken for invaded leaves to move from their normal to their final reflexed position, are given in Table I. Data were obtained from the examination of fifty tomato and thirty potato plants, plants of each group being of comparable size and vigour. They were grown under cool glasshouse conditions.

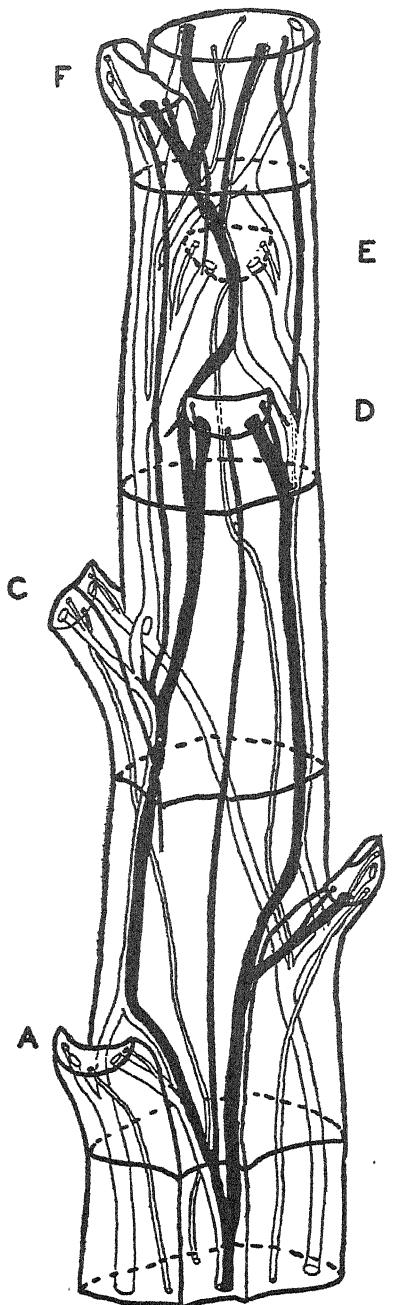
TABLE I
Time Relations of the Epinastic Growth Reactions

Plant.		Minimum time in hours.	Maximum time in hours.	Average time in hours.
Tomato . .	:	5	40	24
Potato . .	:	3	12	5

It is of interest to note that the growth reaction is more rapid in potato than in tomato. A further point noted was that the incubation period for epinastic response in leaves at comparable heights on the stem was considerably shorter for the potato plants than it was for the tomato plants.

V. THE LOCATION OF BACTERIA IN THE VASCULAR BUNDLES IN RELATION TO EPINASTIC RESPONSE

The path of invasion in relation to epinasty was determined by a combination of leaf-sectioning and stem maceration methods after the infected plant had been placed with its cut roots in eosin or methylene blue. The details of the technique have been described elsewhere (Grieve, 1936b), and one example will suffice to show its application here. A photograph of a potato plant which had been inoculated in a lateral fibrous root near the base of the stem some days earlier, is shown on the right of Pl. XX, Fig. 4. A control plant is on the left. Leaves B, C, F, G, H, and I of the infected plant are showing characteristic response, while D is hanging limply with wilted leaflets



and E appears normal. Leaf F shows unilateral wilting of leaflets besides epinasty. Leaf A (not shown in photograph as it was below the level of the top of the pot) was normal. Sections at the bases of the leaves showed invasion of a lateral trace in leaf B, of a lateral and a central trace in leaves F, G, and H, of all traces in leaf D, while no bacteria could be seen in leaves A, C, or E. The path of invasion determined by the maceration method is shown in the diagram. It explains why no bacteria were found at the base of the reacting leaf C, it being an example of action at a distance of 1 centimetre, and why A and E remain normal, no bacteria being present in the leaf traces leading to them. Numerous observations on the position of bacteria in relation to epinasty in a large number of tomato and potato plants, naturally and artificially infected in root and stem, showed that no one condition of invasion was exclusively associated with the phenomenon of epinastic response (see Table II).

In order to ascertain precisely the condition of invasion leading to epinastic response, a series of artificial inoculations of individual leaf-trace bundles was carried out. The path of the bundles could be seen fairly clearly in a tomato plant examined in front of a bright light, and could be judged in potato from a knowledge of their course in the stem. It was found for both plants that invasion of one large lateral trace alone and of a median trace alone suf-

Diagram to show the course of the bundles in the stem of a potato plant, and the path of invading bacteria. Drawn from a potato plant (see Pl. XX, Fig. 4) after staining and maceration treatment.

ficed to induce epinastic response, the degree of epinasty in the latter instance, however, being less marked. Infection of a large lateral trace together with the median trace produced epinastic response equal in degree to that obtained by invasion of the lateral trace alone. Infection of both large lateral traces caused epinastic response, but it was rarely found that the degree of invasion was the same in both laterals. Attempts to show by artificial inoculation of all traces leading outwards to a leaf that wilting without epinasty would ensue as instanced in Pl. XX, Fig. 4, were unsuccessful owing to differential rates of invasion. The reason for this is still obscure, as equal amounts of inoculum from

TABLE II

Plant.	Bacteria in lateral trace only.	Bacteria in one lateral and in median trace.	Bacteria in both lateral traces. <i>a.</i>	Bacteria <i>b.</i>	Bacteria in median trace only.
			Heavy invasion of both.	Heavy invasion in one, light in other.	
Tomato	70 leaves	36 leaves	No cases observed.	8 leaves	1 leaf
Potato	57 ,,	25 ,,	5 leaves	10 ,,	4 leaves

the same source were pricked into the selected bundles in a large number of experiments. Whatever the cause, it is one which operates also in cases of natural or artificial inoculation of lateral roots on all sides of the plant, and also when all three stem bundles are inoculated, since experiments have shown that this differential rate of movement and growth of the invading bacteria is of common occurrence. Were the rates of growth and movement of the organism the same in all the bundles epinasty of leaves would rarely occur.

A study of the degree of penetration and of numbers of the bacteria in leaf traces when epinasty was occurring, failed to show any constant relationship. The following conditions were recorded: (*a*) bacteria might in some cases be present in considerable numbers as far as the terminal leaflet; (*b*) they were present up to or just beyond the zone of bending; (*c*) no bacteria were present in the zone of bending.

With regard to (*c*) it was found that in the majority of tomato plants the distance of invading bacteria from the reacting zone might be as much as 1 centimetre. In the potato plant absence of bacteria in the reflexed leaves was still more common; here the leaves might be reflexed even when the bacteria were about 2 centimetres below the zone of rapid growth at their base. Instances have been recorded where the distance was greater. The evidence suggests that in the potato plant, at least, the bacteria may produce epinasty from a distance.

In contrast to the production of leaf epinasty at a distance are those cases

where no epinasty develops when invasion of a lateral, or of a lateral and a median leaf trace has occurred. Examples may be classified in groups:

- (a) Tomato plants in which two or three leaves near the apex fail to react while leaves lower down on the stem show marked epinasty.
- (b) Tomato and potato plants in which no epinasty, but only unilateral wilting of leaves, occurs.
- (c) Tomato plants under six inches in height, the leaves of which wilt without epinasty.

The failure of epinasty to develop in these instances is believed to be related to the rate of bacterial invasion and the turgor of the test plants, and will be considered in a separate paper.

The data so far presented have been concerned with epinastic response arising out of root or basal stem inoculation. To determine whether the same response would occur when bacteria were moving down through the vessels of a leaf, inoculations were made into selected bundles towards the distal end of the leaf. The technique used was to place a drop of a heavy suspension of *B. solanacearum* on the basal part of a lateral leaflet and make an incision there with a razor. The bacteria in the suspension were sucked back into the tracheae by the negative pressure. Inoculated leaves consistently showed epinastic response. Bacteria were found in the reacting region or a short distance from it. In a second type of experiment the organism was inoculated into a stem bundle near the stem apex. In eight experiments epinastic response occurred only once, and then it was limited to a single leaf just below the point of inoculation. Stem sections taken five to six days after inoculation showed, however, that the bacteria were present in vessels of the stem bundles down to root level. The results obtained indicate that the bacteria grow back very rapidly in the stem bundles and do not easily enter the leaf-trace bundles.

VI. THE INFLUENCE OF ENVIRONMENTAL FACTORS ON EPINASTIC RESPONSE

1. Light.

Both tomato and potato plants on being placed in darkness after inoculation developed leaf epinasty to as great a degree as similar plants held in light after inoculation. The presence of light is not necessary for the reaction. On the other hand, it has been found (unpublished data) that light intensity, other conditions, i.e. temperature and humidity, being constant, can act as a limiting factor on epinastic response through its influence on transpiration and turgor of potentially reactive leaves.

2. Temperature.

Experiments showed that typical epinastic response occurred at temperatures between 64° F. and 80° F., provided that the light intensity was kept low (300–500 foot-candles). Between 80° F. and 90° F. the rapidity of invasion was such that wilting of leaves without epinasty occurred even under low light intensities. The effect of lower temperatures was tested during the

autumn and winter months. During the course of the autumn experiments the average day temperature was approximately 56° F., while the average light intensity was 300 foot-candles. The incubation period of epinasty was found to be very long, but when the reaction did occur, it was quite comparable in degree to that seen in plants infected during the summer months. Plants inoculated during the late autumn and early winter failed to show symptoms, and sectioning revealed the fact that no infection had occurred. The average day temperature was 49° F. and the average light intensity 200 foot-candles. Little growth of plants took place during the period of the experiment. It is apparent from these latter results that the temperature must be high enough to allow reasonable growth of the host plant and of the invading organism, for epinastic response to occur.

3. *Humidity.*

Inoculated plants regularly showed epinastic response both under normal and high light intensities when grown under conditions of high humidity at an average temperature of 70° F. Humidity exercises its effect on the reaction indirectly through the reduction of transpiration and the increase in turgor of the plant.

VII. GRAVITY IN RELATION TO EPINASTIC RESPONSE

Since bacterially induced epinasty occurs against the force of gravity, it was of interest to determine whether the occurrence of the reaction was affected by the neutralization of gravity (plants rotated on a klinostat), or by the additive effect of geotropism and epinasty (plants inverted). Potato plants were used, since epinastic responses occurred most constantly in them, and the time of their appearance could be predicted with reasonable certainty. The following procedure was adopted. Four healthy plants were first rotated on a klinostat, in order to ascertain the degree of epinasty which would develop, and the time necessary for recovery when they were placed in the upright position. They were then inoculated and rotated continuously during the period in which bacterial epinasty was expected to develop. After this they were replaced upright on the bench. It was found that the degree of epinastic response on rotation, was not significantly different in the healthy and in the invaded plants. On return to the upright position, only some of the leaves of the inoculated plants recovered, and sectioning revealed the presence of bacteria in the petiolar bundles of those which remained reflexed.

Four healthy potato plants were next inverted in relation to gravity and the changes in the angles of the leaves measured. All leaves returned to their normal position on being placed upright. Three days after inoculation these plants were again inverted and staked in a vertical position during the incubation period for bacterial epinasty. No measurable increase in angle of leaves occurred over those previously recorded, and on returning them to the upright position several leaves assumed the normal position, while others

remained reflexed. On sectioning, bacteria were found to be present in the lateral traces of those petioles which remained reflexed. The results show that in the presence of invading bacteria, the epinastic movement induced by rotating plants on a klinostat, or inverting them in relation to gravity, is made permanent. Since the angles made with the stem by petioles in invaded plants rotated or inverted in relation to gravity, were within the range of angle variation shown by petioles of invaded plants placed in the normal upright position, there is no evidence, in the case of potato plants, that bacterially induced epinasty is rendered less effective by the neutralization of gravity, or by the co-operation of gravity and epinasty. In their work on ethylene-induced epinasty, Crocker *et al.* (1932), using tomato plants, showed that the presence of ethylene increased the epinasty of the petioles of rotated plants by 30°, and that the ethylene induced two and a half times as much epinastic movement of petioles of upright plants, as of plants rotating on a horizontal klinostat. They further showed that for tomato and African marigold plants, inversion in relation to gravity rendered ethylene practically ineffective. Experiments by the author, using infected tomato plants inverted in relation to gravity, showed that the epinastic reaction failed to occur. Again in tomato plants which were inverted after epinastic response was showing, it was found that in 12 cases out of 15, a decrease in the angle made by the leaf with the stem, occurred. Healthy tomato plants on inversion showed only a slight epinastic response, before torsion effects set in. Experiments similar to those performed by Crocker *et al.* (1932) showed that the weight of the leaf was not a significant factor in the bacterially induced epinasty of tomato petioles. For tomato plants, therefore, it appears that bacteria cannot induce an epinastic growth reaction when the plants are inverted; this is in agreement with the results of Crocker *et al.* (1932) for ethylene. It is clear that there is some fundamental difference between potato and tomato plants in their reactions to gravity, when inverted, and that this has a bearing on the development of epinastic response.

The nature of this difference is at present obscure.

VIII. INVESTIGATION OF POSSIBLE CAUSES OF EPINASTIC RESPONSE

(a) *Mechanical blocking by bacteria.*

Observations had shown that epinastic response occurred when bacteria were present in blocking numbers either close to or in the vessels of the sensitive zone at the base of the petiole. To test whether simple blocking alone was responsible, experiments were performed in which melted cocoa butter (coloured with Sudan III) held at 23° C. was injected or sucked up into the vessels of one or more leaf traces. The efficacy of the cocoa butter as a blocking agent was proved by the inability of dyes to pass the injected region, and sections showed that the cocoa butter could move a distance of several centimetres from the injection point in the stem bundles into the sensitive zone,

and cause blocking there. In the first series of experiments twenty petioles were injected, one lateral trace of each being blocked. A comparable number of petioles was injected with water. No significant change occurred in the angles of the leaves of the water-injected plants, but in three leaves blocked with cocoa butter angle changes of 30° , 20° , and 15° were recorded during the first 24 hours. In further experiments 3 per cent. agar and 10 per cent. gelatin as well as cocoa butter were used as blocking agents, but no significant angle

TABLE III

Experimental plant.	Method of uptake.	No. of plants tested.	Solution injected.	Result.
Tomato . . .	Capillary tube and petiole uptake	34	Precipitate in water	No wilting; no epinasty.
Potato . . .	" "	6	" "	No wilting; no epinasty.
African marigold . . .	" "	10	" "	No wilting; temporary epinasty shown in three cases.
Castor-oil plant . . .	" "	2	" "	No wilting; no epinasty.

Equivalent numbers of control plants injected with boiled precipitate in water showed neither epinasty nor wilting.

changes were recorded. It is within the bounds of possibility that the angle changes recorded above in three instances occurred because some special set of conditions—perhaps of blockage of phloem as well as xylem—was satisfied, but the negative results obtained in over forty experiments, lead to the conclusion that mechanical blocking of vessels does not induce the response.

(b) *The production of a substance, or the setting up of a condition, as a result of bacterial metabolism in the xylem vessels.*

i. *Toxin production.* The production of toxic substances by *B. solanacearum* has been advanced by certain workers (Hutchinson 1913; Nakata, 1927) as causing wilt symptoms, so the possibility of such substances being concerned with the induction of epinastic response had to be considered. Accordingly Hutchinson's mode of experimentation was followed. The organism was grown on nutrient broth for fourteen days, then alcohol was added until no further precipitate came down. The precipitate was filtered off and taken up in sterile water, after which it was fed into the test plants by means of capillary pipettes. A similar solution was boiled before feeding into test plants, and the results are set out in Table III. No evidence of a toxin or any toxic substance capable of inducing epinastic response was obtained by this method. It may also be noted in passing that the wilt effects claimed by Hutchinson for tobacco were not observed in the four host plants examined, namely potato, tomato, castor-oil, and African marigold plants. Evidence from other experiments, notably those on transpiration and absorption in diseased plants (unpublished

data), indicated that *B. solanacearum* does not produce toxins in the host plants tested.

2. *Ammonia production.* Using the test of Molisch (1913), it was demonstrated that ammonia was present in considerable amounts in the vessels of invaded plants while its presence in vessels of healthy plants could be detected only with difficulty. Its possible action in inducing epinastic response was tested by placing stem and petiole parts of tomato plants in buffer solutions, to which sufficient dilute ammonia water had been added to bring the pH to

TABLE IV

*Comparison of the pH Values of Xylem Exudate and of Expressed Sap from Healthy Plants and from Plants Inoculated with *B. solanacearum**

	Tomato.			Potato.		
	Indicator.		Litmus paper.	Indicator.		Litmus paper.
	Liquid (Average value.)	Liquid (Average value.)		Liquid (Average value.)	Liquid (Average value.)	
Healthy plants	Sappy exudate from xylem Expressed sap	Acid Acid	5·6-6·0 5·6-6·0	Acid Acid	5·8-6·0 5·6-6·0	
Plants showing petiole-epinasty	Sap + bacteria exudate	Slightly alkaline after being in contact with cut petiole for about ten minutes	6·8-7·0	Alkaline after being in contact with petiole for about ten minutes		6·8-7·0
	Expressed sap	Acid	6·7-6·9	Acid		6·2-6·6
Plants badly infected and wilted	Sap + bacteria Expressed sap	Alkaline Slightly alkaline	7·2-7·4 7·0-7·2	Alkaline Slightly alkaline		7·2-7·4 7·0-7·2

The liquid indicators were solutions of chlorophenol red, bromocresol purple, and bromothymol blue.

7·4, but no response occurred. When dilute ammonia water in buffer solutions was injected into stem bundles of tomato plants, the leaves showed only wilting effects.

3. *Hydrogen-ion effects.* Tests of the pH of sap from invaded xylem vessels in reacting leaves showed that it tended to be alkaline compared with that from non-invaded vessels and from healthy plants (see Table IV). As indicated in the table two methods of testing the pH of tracheal sap were used. The litmus-paper test is considered the more reliable as it demonstrated the alkalinity due to the ammonia. When a strip of blue litmus paper is first placed against the cut surface of a petiole showing epinastic response it reddens; later a change back to the original blue colour occurs in that part of the strip covering an invaded bundle. Similarly a strip of red litmus paper placed against the cut surface of a reacting petiole, developed a faint blue colour over an invaded

bundle after a time-lag of five to ten minutes. It is clear that the reaction of the tracheal sap is at first obscured by the acid reaction of the contents of cut cells. In the section tests using liquid indicators the effect due to the ammonia is largely lost, and consequently the results obtained err on the side of acidity. To test whether the change in pH due to invasion was causally related to epinastic response, pieces of tomato stem with attached petioles were placed in buffer solutions (KH_2PO_4 —NaOH) over a pH range of 7.0 to 7.6. No epinastic response occurred in any solution over this range, so that it would appear unlikely that an alkaline reaction *per se* is concerned. It is worth recording, however, that the opposite condition of leaf hyponasty could be induced by buffer solutions of pH 4.2 and 4.6 (phthalate—NaOH).

4. *Growth substance production.* It has been shown that heteroauxin is produced by certain fungi and bacteria in culture media (Boysen-Jensen, 1931; Thimann, 1935). Experiments were designed to test its possible production by *B. solanacearum*, both in media and in the host plant. The organism was grown for fourteen days in pure culture in the following medium:

Glucose	2 per cent.
Peptone (Witte's)	1 "
KH_2PO_4	0.05 "
MgSO_4	0.05 "
FeCl_3	trace "
Glass-distilled water	1 litre

The organisms were filtered off, using a Seitz E. K. filter, and the medium concentrated to 1 per cent. of its volume. A control flask of sterile medium was also concentrated to 1 per cent. of its volume. The ether extraction procedure of Thimann and Bonner (1933) was followed and the final water solutions of each tested for growth substance. In the case of the medium in which the organism had grown, a positive test for the presence of growth substance was obtained by the *Pisum* test (Went, 1934), and also the leaves of young tomato plants developed epinasty when placed in the solution. The substance isolated gave all the tests for heteroauxin, being heat-stable and soluble in ether, and it gave the red coloration with ferric chloride and hydrochloric acid.

To determine whether heteroauxin was being produced in the vessels of invaded plants, stem and petiole parts were placed in small phials containing sterile distilled water. The cloudy liquid obtained was filtered free of bacteria, concentrated, and extracted with ether. Negative results were obtained when the extract was taken up in water and tested on healthy plants. The possibility remains that oxidase enzymes, diffusing from the cut stem surfaces, inactivated any growth substances present.

IX. DISCUSSION

The question of the proximate cause of the epinastic response has been investigated having regard to the following possibilities: (a) that mechanical blocking alone, either in the reactive zone or in the leaf traces below it,

initiated the response; (b) that as a result of its growth in the xylem the organism produced some substance, or condition, which disturbed the normal growth of the leaf. No positive answer has been obtained to either of these questions, and the first, in fact, may be considered to be eliminated as a cause. The possibility still remains, however, that with improvements in technique the answer to the second question may be modified. Thus, for example, in work on auxin relations in leaves of tomato plants it has been found that the diffusion method into agar is not satisfactory owing presumably to inactivation of auxin at the cut surfaces by oxidizing enzymes. It may well be that in the experiments reported above, where the bacteria and solutes were allowed to diffuse from cut stems and petioles into water, inactivation of both auxin and bacterially produced heteroauxin occurred. It is hoped to present information on this point at a later date.

Passing to the physiology of the epinastic response we may say that practically nothing as yet is known regarding the normal growth-controlling mechanism at the base of the petiole. The delicacy of its balance is reflected, however, by the fact that ethylene in 1 part per 10 million of air causes the induction of epinasty, and high dilutions of synthetic heteroauxin do the same. In the light of the hormone theory of growth (Went, 1928), and of the auxin distribution experiments of Boysen-Jensen (1936), it would appear logical to suggest that the normal petiolar position is conditioned by some balanced hormone mechanism and that the disturbance of this leads to epinastic response. A study is being made at the present time of auxin distribution in healthy tomato plants with special reference to the petioles, and it is hoped later to examine bacterially induced and other epinastic responses in the light of this work.

SUMMARY

1. A detailed study has been made of epinastic response in leaves of plants invaded by the vascular parasite *Bacterium solanacearum* E.F.S. It has been shown to be an irreversible growth reaction and has been demonstrated to occur in infected potato, tomato, castor-oil, and African marigold plants.
2. Invasion of one lateral leaf-trace bundle, or of a central leaf-trace bundle, suffices to bring about the epinastic response.
3. No constant relation exists between the number of bacteria present at or close to the reactive zone and the onset of epinasty, or between the degree of penetration of the bacteria into the leaf-trace bundle and the onset of epinasty.
4. The growth reaction has been shown to be conditioned by the plant variety, the size of the plant, environmental conditions, and by the relation of the plant to gravity.
5. Possible causes of the epinastic response have been examined, and the following possibilities eliminated: (a) mechanical blocking of the vessels by bacteria; (b) ammonia production and pH effects in the vessels; (c) toxin production. It has been shown that a growth substance—heteroauxin—is

produced in culture media in sufficient amount to induce epinasty in tomato leaves, but so far its presence has not been demonstrated in the invaded vessels.

6. A possible mechanism of the epinastic growth reaction is suggested.

In conclusion the author desires to express his appreciation of the advice and interest accorded to him during this investigation by Dr. McLennan of Melbourne University and by members of the staff of the Botany School, Cambridge.

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EXPLANATION OF PLATE XX

Illustrating Dr. Grieve's paper on 'Epinastic Response Induced in Plants by *Bacterium solanacearum* E.F.S.'

Fig. 1. Apical region of an inoculated potato plant showing the extreme degree of epinastic curvature which may occur in a leaf.

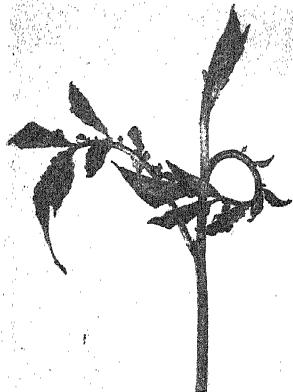
Fig. 2. Tomato plant in which the petioles have been marked with India ink lines, 2 mm apart, on both upper and lower surfaces, before epinasty developed. The greatest growth increase on the upper side is seen to occur between 4 and 6 mm. from the base of the petioles.

Fig. 3. Tomato plant 9 in. in height showing typical epinastic response of leaves due to bacterial invasion.

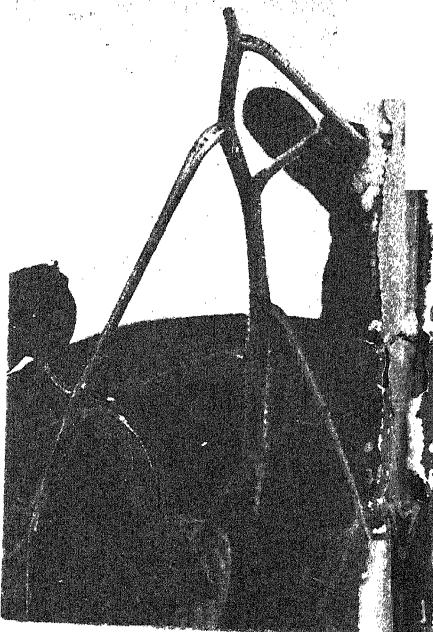
Fig. 4. Inoculated potato plant on right showing epinasty and wilting of leaves. The various conditions shown are discussed in the text.

Fig. 5. Photograph of a transverse section at the base of a reflexed petiole of a tomato plant. Heavy unilateral invasion has occurred. $\times 20$.

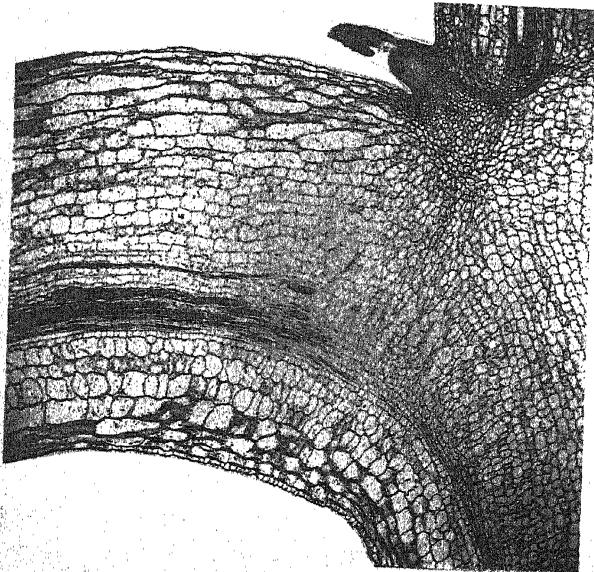
Fig. 6. Photograph of a longitudinal section through a reflexed petiole of a tomato plant, showing the elongation of cells on the upper side. Bacteria are present in the vessels of the vascular bundle. $\times 20$.



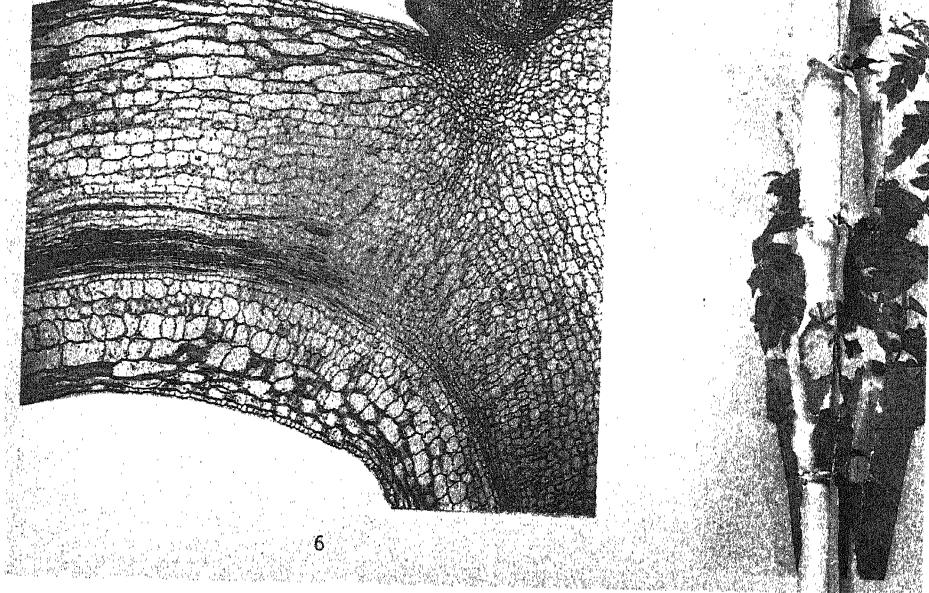
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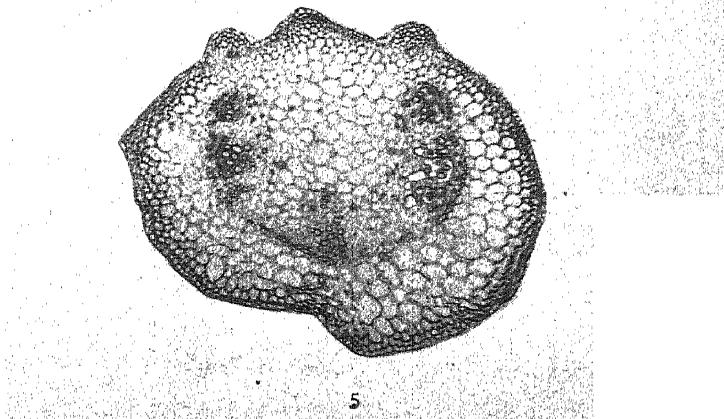
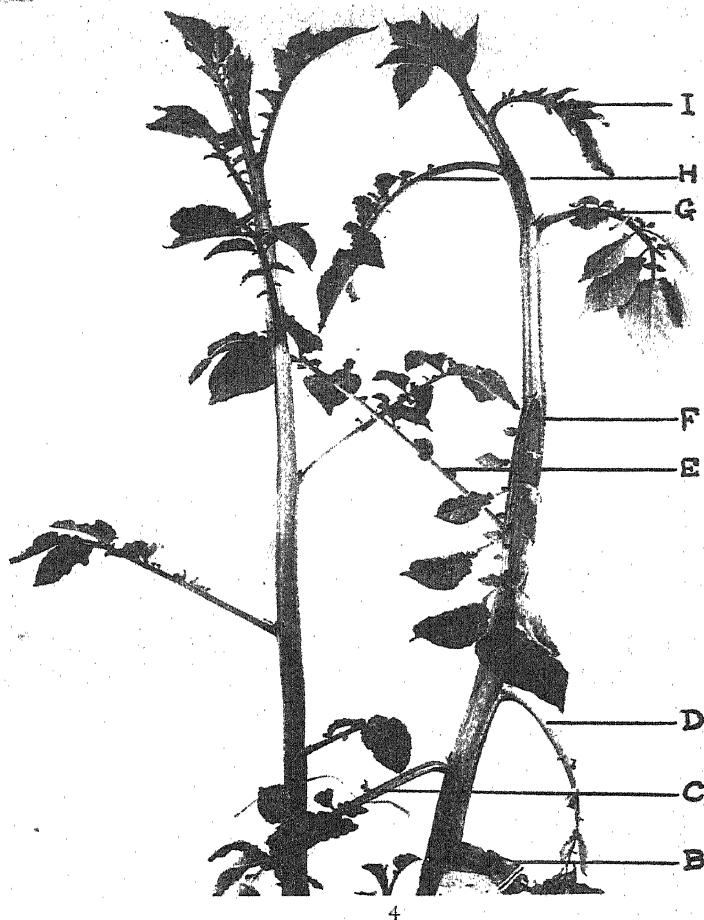


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6





The Influence of Light upon Nitrogen Metabolism in Detached Leaves

BY

W. H. PEARSALL

AND

M. C. BILLIMORIA

With two Figures in the Text

IN a preceding paper (1938) we have described a method by which the changes of protein content in detached leaves could be studied. The present contribution deals with the effects of light upon such leaves and, in particular, upon their nitrogen metabolism.

METHODS

The technique used is developed from that of the earlier paper and involves maintaining leaves under such conditions that balance sheets can be constructed of the materials used. Bacteria-free solutions and pure culture methods are therefore necessary. The success of the method depends upon finding leaves which can be sterilized. It was necessary also to use relatively small amounts of tissue. Previous work had shown that daffodil leaves possessed the qualities required. The leaves were obtained from a large and uniform batch of the single flowered form of *Narcissus pseudo-narcissus* growing semi-wild in a garden. Not less than fifteen leaves, uniform in size, were used in each experimental batch. Each leaf was divided into four segments: the white meristematic base, the yellow green extending zone, the lower grey photosynthetic zone, and the green apical region, the oldest part of the leaf. Each segment was then carefully cut longitudinally into two similar portions. Four similar pairs of samples were thus obtained, weighed, and one of each pair was immediately prepared for analysis to give the original condition of the samples. The remaining four samples were floated on four similar nutrient solutions for three days and their composition determined at the close of the experiment. The nutrient solution contained 0.2 per cent. ammonium nitrate and 3 per cent. glucose as described in the earlier paper (1938). In order to decrease the chance of bacterial infection, the solution was buffered to pH 5.4 with potassium hydrogen phosphate buffers (concentration 0.1 M). Analytical reagents were employed. The leaf material was sterilized by washing with 6 per cent. calcium hypochlorite solution.

(Wilson, 1915) for ten minutes, and then rinsed in sterile distilled water. The nutrient medium was autoclaved for ten minutes at 12 lb. pressure. Flat-bottomed conical flasks were used for the culture media and aseptic precautions employed throughout. No contamination of the experimental material ensued under these conditions in five days. The experiments were limited to three days.

Temperature was regulated by keeping the culture flasks in large glass vessels through which a stream of water circulated. Occasional small differences in temperature between dark and light culture media were observed, the dark medium being occasionally as much as 0.6° C. higher. The cultures in light were illuminated by two 100-watt daylight lamps, one above and one below the cultures. The light intensity (as measured by a Weston photronic cell) was equal for each culture in a series, and it was estimated at between 50 and 1,150 mc.

As it was necessary to use relatively small samples of leaf tissue, division of the samples in order to obtain dry weights introduced rather large sampling errors. For this reason the samples were dried as a whole, by heating to 95° C. for half an hour to destroy enzymes and to constant weight at 65°. Given good aeration of the oven and rapid preliminary drying, the daffodil leaves turned dry a clear green colour by this method, the changes in protein content not being distinguishable from the sampling error. The dried leaf material was finely and uniformly powdered before analysis. Soluble constituents were extracted with 60 per cent. alcohol (until all pigment was removed). The original plan was to estimate only the major constituents, but in view of the results obtained, some further analyses (e.g. of carbohydrates) were made for information, although the technique may not be suitable for all of these constituents. We have therefore only drawn broad conclusions from some of the results, though they are given fully in the Appendix.

All numerical data were estimated as weights of the named constituent per gm. of *original fresh weight*, this being about the average weight of leaf tissue used per sample. The actual weights are given in Appendix E.

The analytical data were obtained by the following methods. Ranker's modification of the Kjeldahl method was used for Total N and Soluble N, using the Pregl micro-apparatus, with exact standardization of all procedure. Total N was obtained by difference. For the estimation of soluble carbonates, ammonia and nitrate, the alcohol was removed by evaporation and solution made up to constant volume with water. The solution was then treated with saturated neutral lead acetate (1 c.c. to 20 gm. of leaf material) and delead with Na_2HPO_4 , the precipitate being thoroughly washed with water. This extract was used for determinations of sugars, ammonia, nitrate. For the two latter an aliquot of solution was placed in the Pregl stilling flask and the outlet was closed by an acid trap. Three cubic centimetres of N NaOH were then added and the ammonia distilled off with steam N/70HCl. This estimate was compared with a "water blank." The solution

was then cooled, 0·2 gm. of Devarda's alloy added, and the solution heated to the boiling-point for fifteen minutes. Ammonia formed was then steam distilled as before. The blank used in this case was a similar aliquot treated in the same way except that no Devarda's alloy was used. This usually corresponded with a water blank, but its use eliminated the possibility that decomposition of higher compounds of nitrogen might take place on prolonged heating with alkali. The estimation of ammonia as given is open to this objection in theory, but using extracts of daffodils, no appreciable decomposition appears to take place in the ten minutes required for the first distillation. (This is not always the case with extracts from other plants.) With uncleared extracts the ammonia nitrogen by this method agrees with that for cleared extract, but the 'Devarda nitrogen' is higher. Extracts from normal daffodil leaves contain no nitrates. All estimates of forms of nitrogen were carried out in triplicate. Similar methods were employed for analysis of the external medium.

Sugars, when determined, were estimated in extracts by the Hagedorn-Jensen method (1923) and in the external solutions by Hane's modification of this method (1929). For the estimation of total sugars, mild hydrolysis with 2·5 per cent. hydrochloric acid for ten minutes at 65° C. was employed (Hulme and Narain, 1931). The acid was neutralized with solid sodium carbonate, with bromothymol blue as indicator.

For starch, about 0·1 gm. of residue was heated with 50 c.c. water on a boiling water bath for one hour, cooled and acidified with two drops of 5 per cent. acetic acid. Then 3 c.c. 0·3 per cent. taka-diastase was added, and the mixture incubated for 3 days at 38° C. in the presence of a little toluene. The mixture was then boiled and cleared as before, hydrolysed as for total sugars and the sugar estimated as glucose (Bish, 1929). All carbohydrates are calculated as glucose.

In assessing the significance of the results it is mainly important to know what variation may be expected between the values of the same fraction, as estimated on the two different half-leaf segments. Estimates of protein N may differ by 3 per cent. of the mean, as shown in the earlier paper (1938). Estimates of total N and soluble N appear to show less divergence but we assume the same figure. The other soluble forms of nitrogen show an agreement of the same order. Estimates of the ammonia N, nitrate N, and total N in the external solution agree to within 2 per cent. of the known value for the original solution, or for known materials added to an experimental solution. Taking this figure on the amounts of nitrogen originally present, it gives a possible variation of 1 mg. in the estimates of 'nitrogen absorbed'. The total N of the external solution by Ranker's method is usually somewhat less than the sum of nitrate and ammonia N, owing to the difficulty of reducing large proportions of nitrate in the presence of sugar, but the 'nitrogen absorbed' estimated by this method is within 1 mg. of the other estimates.

Three series of experiments are discussed in this paper. The first was

carried out with leaves of plants whose flower stalks were not or barely visible and at a temperature of $16 \pm 1^\circ\text{C}$. In two later series, older plants from the same batch were used. In Series 2 flowers were opening and in Series 3 the flowers were fully mature with signs of withering. At this stage growth of leaves in length ceased. Both these later series were carried out at $22 \pm 0.6^\circ\text{C}$, with the idea of accelerating the changes observed. The results of Series 1 are directly comparable with those of series described earlier (*loc. cit.*) except for the rather lower pH (5.4 instead of 6.0), and the results are not materially different from those of earlier experiments.

PROTEIN FORMATION IN LIGHT AND DARK

The main problem under consideration was that of determining the effect of light on protein content. It is convenient to examine this first, before going on to analyse the causes which may have contributed to the effects observed. The results as they bear on this problem are summarized in Table I.

TABLE I
*Gain or Loss of Protein N and of Dry Weight, as Percentages
of the original Amounts*

Series	Protein N.			Dry Weight.		
	1 Temp. °C.	2 22	3 22	1 16	2 22	3 22
<i>Light</i>						
1 Base	12.2	3.8	8.5	18.5	-6.2	0.8
2	18.8	46.2	30.0	43.5	26.8	25.7
3	-2.5	-0.3	1.5	27.5	11.4	4.4
4 Apex	-5.7	-14.8	-8.35	10.5	-6.6	-17.7
<i>Dark</i>						
1 Base	10.6	-0.6	-6.7	14.3	11.9	-4.2
2	-6.2	-8.4	-18.5	6.5	-1.9	-2.0
3	-2.85	-10.5	-6.1	11.5	-11.0	-17.9
4 Apex	-8.05	-14.5	-16.0	2.6	-14.4	-30.6

As each estimate of protein N gain is subject to a possible variation of 3 per cent., the significant differences in comparing any *two* of the figures in this table must be six units of percentage. In the basal segments (1), light has therefore no significant effect on protein content except in Series 3. Light has most effect in the second, elongating segments, where there is a clear tendency towards hydrolysis in the dark, but large gains in protein in the light. The difference is greatest in Series 2 and 3, probably because of the higher temperature. Changes in protein content are negligible in the third (fully green) segments when illuminated, but Series 2 and 3 show protein hydrolysis in the dark. The fourth (or apical and oldest) segments all show losses, the differences between light and dark being not or barely significant.

The main features are thus the absence of protein gains in either of the

fully green and mature types of segment (3 and 4) and, on the other hand, the very large effects produced in the second segments which are elongating and just becoming pigmented. It is only in these segments that the net gains of protein are markedly increased by light under these conditions. The white basal segments show insignificant light effects except in Series 3, the oldest leaves, and in regard to these it may be advisable to reserve judgement, partly because there is no other confirmatory evidence and partly because of the difficulty of being sure that the basal parts as separated contain no pigment and are not 'extending'. The method of separation by visible signs must necessarily be approximate in regard to the real physiological state of the tissues, and in several cases colour has developed in basal segments during an experiment. Further, in Series 3, the leaves were approaching the stage (after flowering) when growth slows down, and it may be that the basal segments tend to pass over into the extending stage at this period. Other effects which can reasonably be attributed to the greater age of the leaves in Series 2 and 3 are not very pronounced. In view of the results considered later, it is probable that the greater tendency towards hydrolysis of protein in darkness in Series 2 and 3 is due to the higher temperature. The main conclusion is, therefore, that the effect of light on protein content is shown mainly in the zone of cell extension, while protein gains are confined to growing tissues.

Changes in dry weight.

The changes in dry weight, also given in Table I, are of interest in comparison with the changes in protein. Our own estimates show that the possible error in these estimates is of the same order as that for protein N, possibly slightly higher. This agrees with Denny's figure (1930) of between three and four per cent. We assume, therefore, that a difference of eight units is normally probably significant in comparing any two figures in the table. Hence exposure to light increases the dry weight gains or reduces the losses, while a higher temperature increases the tendency towards loss or reduces the gains. Finally, the apical segments in Series 3 show significantly lower figures than those of Series 2, which suggests an effect of age comparable to that shown in any single series of segments.

In comparison with the protein figures, the dry weight changes show less sensitivity to the 'age' differences between the segments (of any one series). Gains may be present in the third segments for example. It should be noted, moreover, that the effect of higher temperature on the gains is quite different in the two cases. A higher temperature increases the protein gains but reduces those of dry weight. It should finally be added that the changes in protein N account for only a small part of the dry weight changes (see Appendix B). It is not, however, very probable that these changes in dry weight are only or mainly due to photosynthesis. It may be seen from Table II that the net gains of dry weight by the tissues are much less than the dry weight losses

from the external solution. This, as well as the large gains in the second segments, which were incompletely pigmented, suggests that the gains are also due to accumulation of dissolved solids from the external medium, a view borne out by the data given below.

ABSORPTION FROM THE EXTERNAL MEDIUM

Water. The data for the amounts of water absorbed are given in Appendix C, but as they do not bear on the general problems involved, they are not discussed in detail in spite of their intrinsic interest. An analysis of the results shows that light greatly increases the rate of water absorption, particularly in pigmented tissues and at the higher temperature. The data for water content also allow estimates to be made of the average concentrations of substances present in the tissues at the close of the experiment, these being given in Appendices C and D and discussed in the text. In the present connexion it may be noted that there is a direct proportionality between the amount of water absorbed in the dark and the original total sugar concentration.

Sugars. The amounts of sugar absorbed from the external medium are given in Table II. In this and subsequent tables the data are given for 10 gm. of original tissue, which is slightly below the average weight of the samples, but which involves the least general change in the results. The estimates of sugar absorbed are subject to a possible error of 110–120 mg. owing to the large amounts of sugar in the external medium. Estimates are also given in Table II of the total dry weight loss (sugar absorbed minus dry weight gains) and these involve an additional possible error of the order of 50 mg., so that we assume a possible variation of 170 mg., although in many cases it may be much less. It is obvious, therefore, that only general conclusions can be drawn from the data.

TABLE II

Sugar Absorbed from the External Medium and Total Estimated Losses of Dry Weight (both as mg. per 10 gm. of original Tissue), also Losses of Dry Weight as Units per Unit of original Protein N

Series 1	Sugar absorbed.			Total loss of dry weight.			Loss per unit protein N.		
	1	2	3	1	2	3	1	2	3
<i>Light</i>									
1 Base	739	564	547	579	615	540	39·3	42·2	40·8
2	534	693	543	116	462	310	5·9	27·3	17·1
3	766	799	679	387	666	623	10·2	18·4	16·2
4 Apex	744	989	647	557	1091	960	9·2	18·5	17·9
<i>Dark</i>									
1 Base	473	721	396	334	625	431	24·2	37·2	29·7
2	293	472	333	230	489	351	12·8	22·3	17·2
3	388	517	487	332	652	711	8·9	18·0	18·2
4 Apex	442	919	509	395	1176	1053	6·3	20·0	19·9

The data for sugars show that in pigmented tissues there is a marked tendency for higher absorption of sugars in the light, the gains being relatively greatest in the second and third segments, in agreement with the figures for dry weight gains considered previously. The large amounts of sugar absorbed seem, however, to be mainly respired, since even when allowance is made for the dry weight gains of the tissue there are considerable total losses. Light has no measurable effect on the total dry weight losses, but these are increased by the higher temperatures of Series 2 and 3, which is to be expected if these losses are considered as measures of respiration. A general feature of all series is that the lowest losses occur in the second elongating segments, equally evident if the data are expressed as a percentage of the dry weight of tissue used. This effect, however, is probably related to the very different protein contents of the different segment types. If the protein content is a measure of the proportion of protoplasm (as is frequently assumed), the loss per unit of original protein N will give a better estimate of the relative intensities of respiration in the different segments. The figures for this estimate are, therefore, given in the last columns of Table II, to draw attention to the possibility that the high dry weight losses in the apical segments appear to be related to their high protein contents. Otherwise these figures simply confirm the general conclusions drawn from the total dry weight losses. None of these results offer any clue as to the cause of the increased protein synthesis in light.

Nitrogen. Considering next the absorption of nitrogen from the external solution, the results are given in Table III. A significant difference between any two results is probable if they diverge by more than the values given in the last column.

TABLE III
Amounts (mg.) of Ammonia N and Nitrate N Absorbed by 10 gm.
of original Tissue

Series	Ammonia—N.			Nitrate—N.			Significant differences.
	16	22	22	16	22	22	
<i>Light</i>							
1 Base	7.75	6.7	4.1	6.7	8.5	8.0	1.5
2	9.2	10.2	8.3	9.8	14.1	12.6	1.3
3	14.15	10.4	10.2	14.3	16.8	17.2	1.7
4 Apex	16.85	11.1	10.8	16.3	18.2	18.6	2.7
<i>Dark</i>							
1 Base	5.4	7.55	4.4	4.3	9.45	6.3	1.5
2	5.6	6.6	6.4	3.6	7.6	6.6	1.3
3	9.3	7.85	6.3	5.7	9.0	8.3	1.7
4 Apex	11.3	8.4	3.8	5.0	11.3	8.4	2.7

It is apparent that the absorption of both ammonium and nitrate ions is usually increased by light. Further, light increases nitrate absorption relatively more than that of ammonium ions. These effects, however, are not

consistently shown in the basal, meristematic segments, but are chiefly confined to the pigmented tissues (2-4). In these pigmented segments also a higher temperature tends to increase the rate of nitrate absorption but to reduce the rate of ammonium ion absorption. It is possible, therefore, that the light may raise the internal temperature of the illuminated leaves and hence indirectly affect the relative absorption of these ions. Finally it may be noted that the apparent tendency towards increased ion absorption in the older apical segments is related to the higher protein N content of these segments, as was the case for dry weight losses.

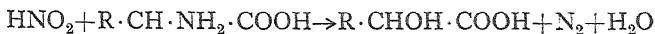
The results, however expressed, do not point to any marked advantage possessed by the second segments in regard to the absorption of substances. Thus while the larger gains in protein in these segments on illumination may well be connected with a greater uptake of nitrogenous substrates, a different explanation must then be invoked to explain the tendency towards hydrolysis shown in the older segments (3 and 4) which may absorb still more nitrogen and sugar in the light. Further, the internal concentrations of inorganic N and sugars are not consistently different either in different leaf segments or in light as compared with darkness (Appendices C and D). In short, the mature segments appear to have lost their capacity to utilize inorganic N in protein formation. This may be due either to a failure of the synthetic mechanism (or of part of it) or else to the existence of other alternative reactions which tend to proceed in older leaves at a greater rate, as discussed below.

A final point worthy of attention at this stage is the relation between the internal concentrations of inorganic nitrogen (at the close of the experiments) and the concentration in the external medium. The original concentration of ammonia N or of nitrate N was only 0·6 mg. per c.c. A reference to Appendix C will show that in almost every case the approximate internal concentrations of these fractions were greatly in excess of this figure at the close of an experiment. The nitrate N was wholly accumulated during the experiment. The ammonia N, however, was apparently present even at the beginning in an average concentration higher than that of the external medium.

METABOLIC PROCESSES

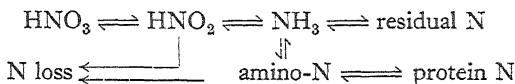
As the various aspects of this work already discussed have thrown no light on the mechanism which may underlie increased protein gains in the light, it now becomes desirable to consider in greater detail the transformations through which the absorbed nitrogen passes in the tissues. It is clear, perhaps, that it is only possible to follow this nitrogen when balance sheets, showing gains and losses by the tissues, can be constructed. This was the main object of the experiments. When, however, the first balance sheets were constructed, an unexpected difficulty was encountered. It was discovered that, when inorganic sources of nitrogen were employed, a variable and often

a large part of the nitrogen absorbed might be lost during the experiment in some form not estimated by the methods employed, and hence, presumably as gaseous nitrogen. Evidence has been given in an earlier paper (1937) to support the following hypothesis as to the mechanism of this nitrogen loss. It is assumed that nitrate is reduced to nitrous acid (Warburg and Negelein, 1920). The nitrous acid formed then combines with mono-amino-N to form elementary nitrogen, which escapes.



This is the basis of the Van Slyke method for estimating amino-N and, as is well known, the reaction is extremely rapid.

We assume in this paper that the observed N losses are caused by the reaction indicated above and we accept from the earlier paper (1937) the following scheme of nitrogen metabolism in these tissues.



As to possible connexions between the amino N and other forms of soluble organic N (called 'residual N') no assumptions are made except that both may arise from ammonia N and that through this substance they are interchangeable.

The important point arising from this scheme is that, if we assume the mechanism of N loss to be that postulated above, we can still examine approximately the paths along which the absorbed N passes, for half the N loss will represent nitrite N and the remaining half will represent amino N. This is merely the quantitative implication of our first assumption; and it forms the necessary basis of much of the discussion following in the next sections. By utilizing this we obtain the figures for important fractions which are given in Tables IV and V and in Appendix B. In these tables the estimated significant differences between figures for segments of the same type tend to be closely similar, but as they vary with the total N content of the segments they are larger in the apical segments. To avoid repetition and multiplication of tables we give the largest significant differences for each segment class and for any pair of results on the corresponding line. These are calculated from the data given earlier (p. 603). In comparing results on different lines in the tables, the higher significant difference is usually taken.

Reduction of nitrates and fate of nitrites.

In considering the fate of the nitrogen absorbed, the first step in logical order is the reduction of nitrate to nitrite. The amount converted in this way is given by (total nitrate N absorbed) minus (nitrate N in the tissue) as shown in Table IV. This table also gives N loss, half of which is assumed to represent nitrite N converted to nitrogen.

TABLE IV

Nitrate N Converted and N Loss (mg. per 10 gm. of original Tissue)

Series	Nitrate N converted.			N loss.			Significant differences.
	1	2	3	1	2	3	
Temp. °C.	16	22	22	16	22	22	
<i>Light</i>							
1 Base	3.5	6.1	6.7	3.5	6.6	5.4	3.0
2	6.4	12.1	12.6	4.0	5.2	4.4	3.6
3	10.3	14.9	16.3	12.5	15.7	14.0	4.2
4 Apex	9.8	17.9	18.6	24.4	29.3	27.5	4.7
<i>Dark</i>							
1 Base	1.6	6.0	4.7	2.9	8.4	6.0	3.2
2	0.0	5.4	4.6	3.6	8.5	8.1	3.5
3	1.8	7.0	7.2	5.0	15.1	13.1	4.0
4 Apex	1.7	10.1	7.8	8.3	22.4	21.1	4.5

Significant differences for nitrate N converted range from 2.0 to 2.2 mg.

The table shows that in all series, light accelerates the process of nitrate reduction in pigmented tissues (segments 2 to 4). An increase of temperature also increases the rate of conversion both in darkness and in light. It appears probable that, in darkness, most of the nitrite N thus formed is lost, as the amounts are of the same order of magnitude as 'half the N loss'. In light, however, there may be a considerable excess of 'nitrate N converted' over 'half the N loss', and this appears to be correlated with corresponding gains of organic N shown in Table V. In Fig. 1, showing the relation between 'half the N loss' and the amount of nitrate N converted, the points are arranged in three groups: (i) those from segments showing significant gains of protein, (ii) those from segments showing no significant changes in protein, (iii) those from segments showing net protein hydrolysis. It thus appears from the graph that the N loss tends to be small and constant if gains of protein occur. If no gain of protein occurs the N loss increases less rapidly than the nitrate N converted. If protein hydrolysis predominates the rate of N loss is greatly increased, though evidently it is also limited by the rate of nitrate conversion. Thus, this evidence as to the effect of increased protein hydrolysis upon N loss justifies our assumption as to the mechanism underlying this loss as it appears to be increased by the production of amino-acids in protein hydrolysis.

The comparative figures for N loss are of interest in one other respect. They show a well-marked rise at the higher temperature (Series 2 and 3) in the dark, but in light, increased temperature appears to have no significant effect, and indeed, only in Series 1 (Nos. 3 and 4) and in the apical segments of Series 2 and 3, does light produce any increase in the rate of N loss. This can only mean that N loss tends to be limited (directly or indirectly) by the amount of light energy when the leaves are illuminated at these temperatures.

Formation of organic nitrogen.

As ammonia occupies a central position in nitrogen metabolism and can demonstrably be formed or removed in many ways, it is not a good subject for analytical treatment. But the total amount of ammonia converted to organic N (including that formed from nitrate) can be estimated with reasonable accuracy, and pertinent figures are given in Table 5. In this table the

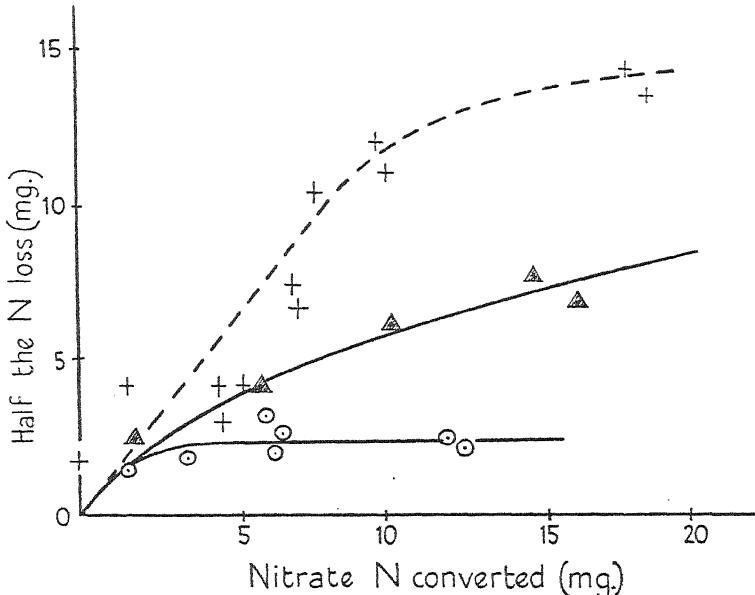


FIG. 1. Showing the relations between the nitrate-N reduced, the N loss and protein synthesis or hydrolysis. ○ Tissues showing gains in protein. △ Tissues without changes in protein. + Tissues showing protein losses.

total organic N produced is obtained as (total N gained by tissue) plus (half the N loss) minus (inorganic N gained by tissue). The *organic N retained* by the tissue is clearly the difference between the gains of total N and of inorganic N. The estimates of *total organic N produced* are logically open to the objection that it is assumed that none of the nitrite N lost is derived from organic N. We believe this assumption to be justified with our daffodil leaves for two reasons: Firstly, no N losses have been observed when organic sources of nitrogen have been used in similar experiments, suggesting that amide or amino N is not easily degraded to nitrite N. Secondly, if in the present experiments such a down-grade source of nitrite had existed, half the N loss would, at times, be significantly higher than the nitrate N converted. This is apparently never the case (Table IV). The evidence available thus suggests that we must assume all the nitrite N to come from inorganic N (cf. Pearsall and Billimoria, 1937).

TABLE V

Production and Retention of Organic N (mg. per 10 gm. original Tissue)

Series	Total organic N produced.			Significant difference.	Organic N retained.			Significant difference.
	1	2	3		1	2	3	
<i>Light</i>								
1 Base	8.2	8.2	5.2	2.8	6.5	4.9	2.5	1.4
2	10.2	15.9	13.2	3.7	8.1	13.3	10.9	2.0
3	15.5	13.8	13.9	4.9	9.2	6.0	6.9	2.9
4 Apex	9.2	10.2	12.7	5.3	-3.0	-4.4	-1.0	3.0
<i>Dark</i>								
1 Base	4.8	7.4	5.5	3.1	3.4	3.1	2.4	1.5
2	2.9	5.3	5.8	3.5	1.1	1.0	1.7	1.9
3	5.9	5.3	5.1	4.2	3.4	-2.3	-1.5	2.3
4 Apex	6.8	4.4	-0.3	5.2	2.7	-6.8	-10.9	3.0
Temp. °C.	16	22	22	—	17	22	22	—

The 'organic N produced', giving the total amount of ammonia N converted to organic forms, represents the most important stage in the metabolic sequence, since it involves the combination of nitrogen and carbon. We know little about the mechanisms involved in this combination, and the figures are therefore especially interesting. They show firstly that light usually accelerates the production of organic N in the pigmented segments though not in the basal ones. The effect is not significant in the apical segments of Series 1. This effect of light has previously evaded reasonable proof. Secondly, the figures offer a problem in that temperature has apparently no effect on the process of organic N formation, even in the dark. Thirdly, there are apparently no definite age effects, except possibly the absence of any formation of organic N in the apical segments of Series 3, dark. It may be noted, however, that the age effects are clearly shown in the amounts of organic N retained by the tissues. The beneficial effect of light on this retention is evident. Otherwise the important feature of these results is the proof that the formation of organic N is accelerated by light.

The more difficult problem is the apparent absence of a temperature effect in organic N production. In the case of illuminated leaves this might be due to the fact that the process was limited by light intensity. This explanation obviously will not hold for leaves in darkness, and, for these, it seems that we must be dealing with a reaction limited either by some physical factor such as diffusion or by a similar factor governing the supply of one of the substrates involved. The limiting effect in darkness must be removed by light. Hence it seems that the simplest explanation of the facts is that the rate of supply of substrate is severely limited in darkness by something offering resistance to its diffusion. This resistance appears to be greatly decreased by exposure to light, though not entirely removed. Possibly tending to support this suggestion is the fact that it is not easy to demonstrate any marked

difference in internal metabolic conditions between the 'light' and 'dark' leaves. The similarity in the internal concentrations of sugars, organic nitrogen, ammonia and nitrate N is quite striking (Appendices C and D) and points rather to a feature of protoplasmic organization as a more probable limiting factor.

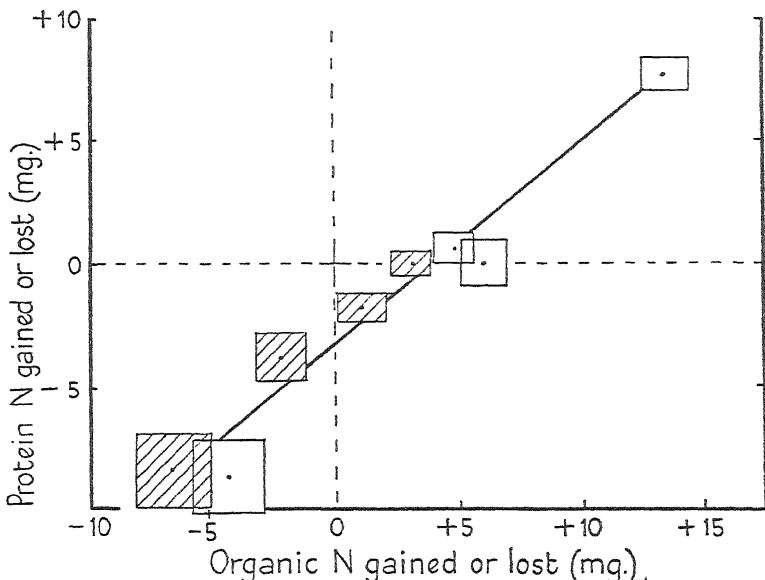


FIG. 2. Showing the relation between changes of protein N and of organic N retained by tissue. Results from 'dark' samples given by shaded squares.

Condensation of protein.

The last stage in the series of metabolic changes is the synthesis of protein from organic forms of nitrogen. We may provisionally assume that this is a process of the type exemplified by the condensation of amino-acids to poly-peptides. It appears unlikely that such a process would be affected by light, and it is difficult in the present evidence to find any justification for such a view. If light has any direct effect on protein formation then it seems possible that a larger proportion of the organic nitrogen gained by the tissues would be converted to protein in the light, while in the dark a larger proportion would remain as soluble organic nitrogen. In fact, however, the data show that no such effect of light can be detected. The graph illustrating this (Fig. 2) is based on the results of Series 2 which cover the greatest range of protein gains and losses. The suggestion applies to all results.

CONCLUSIONS

It thus appears that light may demonstrably affect the nitrogen metabolism of pigmented leaves in three ways, firstly, by increasing the rate of absorption

of inorganic N, secondly, by accelerating the process of nitrate reduction and, thirdly, by increasing the rate of production of organic N. Light may increase the rate of nitrogen loss at the lower temperature (16° C.) used but, at the higher temperature, N loss tends to be limited by some factor such as the rate of organic N formation, and by the extent to which this organic N is converted into protein. Further, because ammonia is in excess and accumulates in the tissues, it appears possible that the rate of organic N formation may be the real limiting factor in nitrogen metabolism under these conditions of experimentation, although the fate of the organic compounds formed may be very different in different parts of the leaves.

The capacity of the different leaf segments to gain protein depends in part at least upon their age, and may be also connected with their ability to retain the organic N made. The results suggest that the fate of the organic N depends upon whether protein is gained or not. If not, the organic N tends to be lost in a detached tissue, this loss being increased when protein losses occur. The factors determining whether protein is gained or lost appear to be different from those which influence the other stages of the N metabolism.

We acknowledge, with pleasure, valuable criticisms received from Mr. G. E. Briggs.

APPENDIX A

Series I. Nitrogen Fractions (mg. per 10 gm. original Fresh Weight)

	Total N.	Sol. N.	Ammonia N.	Nitrate N.	Protein N.	Sol. organic N.
<i>Light</i>						
Original 1 Base	24.28	9.56	1.84	—	14.72	7.72
2	32.98	13.40	2.04	—	19.58	11.36
3	51.62	13.64	2.40	—	37.98	11.24
4 Apex	70.72	10.51	1.37	—	60.21	9.14
Experimental 1 Base	35.23	18.71	3.04	3.27	16.52	12.40
2	47.87	24.63	5.44	3.35	23.24	15.83
3	67.50	30.48	5.05	3.99	37.02	21.44
4 Apex	79.49	22.68	6.65	6.50	56.81	9.53
<i>Dark</i>						
Original 1 Base	23.18	9.37	1.78	—	13.81	7.59
2	30.76	12.87	2.22	—	17.89	10.65
3	49.65	12.26	2.25	—	37.39	10.01
4 Apex	72.57	10.11	1.93	—	62.46	8.18
Experimental 1 Base	30.03	14.75	2.50	2.74	15.28	9.51
2	36.44	19.66	3.15	3.62	16.78	12.89
3	59.66	23.37	5.01	3.87	36.29	14.49
4 Apex	80.55	23.11	3.96	3.26	57.44	15.89

Nitrogen Metabolism in Detached Leaves

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Series II. Nitrogen Fractions (mg. per 10 gm. original Fresh Weight)

		Soluble		Protein	Sol.
	Total N.	N.	NH ₃ N.	NO ₃ N.	organic N.
<i>Light</i>					
Original 1 Base	24.91	10.34	1.37	—	14.57 8.97
2	30.23	13.29	1.57	—	16.94 11.72
3	48.03	11.70	2.01	—	36.33 9.69
4 Apex	68.86	9.78	1.36	—	59.08 8.42
Experimental 1 Base	33.51	18.38	2.73	2.38	15.13 13.27
2	49.40	24.65	5.46	2.00	24.75 17.19
3	59.51	23.29	5.60	1.92	36.22 15.77
4 Apex	68.87	18.52	5.45	0.36	50.35 12.71
<i>Dark</i>	*				
Original 1 Base	29.71	12.90	1.98	—	16.81 10.92
2	35.96	14.04	1.80	—	21.92 12.24
3	48.05	11.71	1.48	—	36.34 10.23
4 Apex	67.94	9.25	1.15	—	58.69 8.10
Experimental 1 Base	38.29	21.58	3.98	3.43	16.71 14.17
2	41.83	21.75	4.43	2.21	20.08 15.11
3	49.78	17.26	3.55	1.97	32.52 11.74
4 Apex	65.18	14.88	4.02	1.17	50.30 9.69
<i>Light</i>					
					<i>Series III</i>
Original 1 Base	22.73	9.48	1.43	—	13.25 8.05
2	30.95	12.89	1.81	—	18.06 11.08
3	49.18	10.68	0.67	—	38.50 10.01
4 Apex	62.73	9.05	1.29	—	53.68 7.76
Experimental 1 Base	29.42	15.05	4.31	1.32	14.37 9.42
2	47.38	23.91	7.30	—	23.47 16.61
3	62.60	23.54	6.35	0.88	39.06 16.31
4 Apex	64.70	15.51	4.25	—	49.19 11.26
<i>Dark</i>					
Original 1 Base	23.28	8.82	1.05	—	14.46 7.77
2	31.37	11.00	1.94	—	20.37 9.06
3	50.77	11.71	1.24	—	39.06 10.47
4 Apex	63.11	10.19	1.77	—	52.92 8.42
Experimental 1 Base	28.02	14.53	1.67	1.67	13.49 11.19
2	36.26	19.66	3.14	1.98	16.60 14.54
3	52.30	15.62	3.06	1.17	36.68 11.39
4 Apex	54.14	9.68	3.08	0.61	44.46 5.99

APPENDIX B

Balance Sheets

Nitrogen Fractions (mg. per 10 gm. original Fresh Weight)

Gained by tissues

	N removed from solution.	N lost.	Total N.	Gained by tissues		
				Protein N.	Inorganic N.	Sol. organic N.
<i>Series I</i>						
Light 1 Base	14.48	3.53	10.95	1.80	4.47	4.68
2	18.93	4.04	14.89	3.66	6.75	4.48
3	28.42	12.54	15.88	-0.96	6.64	10.20
4 Apex	33.15	24.38	8.77	-3.40	11.78	0.39

		Gained by tissues				
		N removed from solution.	N lost.	Total N.	Protein N.	Inorganic N.
Dark	1 Base	9.76	2.91	6.85	1.47	3.46
	2	9.25	3.57	5.68	-1.09	4.55
	3	14.99	4.98	10.01	-1.10	6.63
	4 Apex	16.24	8.26	7.98	-5.02	5.29
<i>Series 2</i>						
Light	1 Base	15.20	6.60	8.60	0.56	3.74
	2	24.35	5.18	19.17	7.81	5.89
	3	27.22	15.74	11.48	-0.11	5.51
	4 Apex	29.35	29.34	0.01	-8.73	4.45
Dark	1 Base	17.00	8.42	8.58	-0.10	5.43
	2	14.23	8.46	5.87	-1.84	4.84
	3	16.87	15.14	1.73	-3.82	4.04
	4 Apex	19.68	22.44	-2.76	-8.38	4.04
<i>Series 3</i>						
Light	1 Base	12.07	5.38	6.69	1.12	4.20
	2	20.88	4.45	16.43	5.41	5.49
	3	27.43	14.01	13.42	0.56	6.56
	4 Apex	29.44	27.47	1.97	-4.49	2.96
Dark	1 Base	10.74	6.00	4.74	-0.97	2.29
	2	13.01	8.12	4.89	-3.77	3.18
	3	14.63	13.10	1.53	-2.38	2.99
	4 Apex	12.16	21.13	-8.97	-8.46	1.92

APPENDIX C

Changes in Water and Dry Weight of 10 gm. original Tissue, and Final Internal Concentrations of Nitrogen Fractions (mg. per g. of Water)

	Original.		Final.		Approximate concentrations		
	Water.	Dry weight.	Water.	Dry weight.	NH ₃ N.	NO ₃ N.	Sol. organic N.
<i>Series 1</i>							
Light 1 Base	9.135	0.865	12.802	1.025	2.1	2.2	8.4
2	9.039	0.961	16.832	1.378	3.2	2.0	9.4
3	8.629	1.371	17.713	1.749	2.85	2.3	12.1
4 Apex	8.198	1.802	17.575	1.991	3.8	3.7	5.4
Dark 1 Base	9.135	0.865	10.711	0.989	2.3	2.6	8.9
2	9.039	0.961	11.326	1.023	2.8	3.2	10.5
3	8.629	1.371	13.989	1.529	3.6	2.8	10.4
4 Apex	8.198	1.802	16.030	1.848	2.5	2.0	9.0
<i>Series 2</i>							
Light 1 Base	9.178	0.822	10.027	0.771	2.7	2.4	13.3
2	9.116	0.884	12.432	1.121	4.4	1.6	13.8
3	8.836	1.164	12.506	1.327	4.5	1.5	12.6
4 Apex	8.283	1.717	12.898	1.604	4.2	2.8	7.3

	Original.		Final.		Approximate concentrations		
	Water.	Dry weight.	Water.	Dry weight.	NH ₃ N.	NO ₃ N.	Sol. organic N.
Dark 1 Base	9.180	0.820	10.888	0.979	3.65	3.15	13.0
	2	9.104	0.896	11.255	0.879	4.0	2.0
	3	8.852	1.148	10.872	1.022	3.3	1.8
	4 Apex	8.268	1.734	11.319	1.485	3.7	1.0
<i>Series 3</i>							
Light 1 Base	9.196	0.804	10.756	0.909	4.0	1.2	8.8
	2	9.092	0.908	13.264	1.155	5.5	0.0
	3	8.788	1.212	14.183	1.266	4.5	0.6
	4 Apex	8.225	1.775	13.774	1.460	3.1	0.0
Dark 1 Base	9.187	0.813	10.539	0.779	1.6	1.6	10.6
	2	9.107	0.893	11.399	0.875	2.7	1.7
	3	8.765	1.235	12.398	1.013	2.5	0.9
	4 Apex	8.241	1.759	11.778	1.537	2.9	0.6

APPENDIX D

Carbohydrates (mg. per 10 gm. of original Tissue) and Internal Sugar Concentrations (mg. per gm. of Water in Final Tissue). Series 1

			Total carbo-hydrate.	Titra-table acid.*	Total sugar concentration.
<i>Light</i>					
Original 1 Base	170	13	143	4.96	20.0
2	163	31	31	5.81	21.4
3	139	110	91	6.07	28.8
4 Apex	131	246	63	6.01	46.0
Experimental 1 Base	194	72	87	9.46	18.0
2	198	155	17	13.80	20.9
3	153	152	137	16.91	17.3
4 Apex	277	124	110	13.56	31.0
<i>Dark</i>					
Original 1 Base	184	10	97	5.16	31.2
2	175	53	27	5.89	25.2
3	151	159	40	5.77	36.0
4 Apex	133	261	63	5.62	48.1
Experimental 1 Base	217	115	88	8.33	31.0
2	183	53	53	9.17	21.9
3	224	100	100	10.09	23.2
4 Apex	282	49	97	10.42	21.3

* As c.c. of N/10 acid per 10 gm. original tissue.

APPENDIX E

Fresh Weight (gm.) of Tissue Used in Each Experiment

Series Condition	Light	¹ Dark	Light	² Dark	Light	³ Dark
1 Base	13.280	10.736	15.510	11.791	16.000	16.410
2	16.523	13.917	17.751	15.249	17.505	16.503
3	12.840	11.335	13.765	11.110	12.513	11.791
4 Apex	6.936	5.086	8.850	7.720	9.260	6.875

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The Interaction of Factors in the Growth of *Lemma*

XIV. The Interaction of Potassium and Light Intensity in Relation to Growth and Assimilation

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With sixteen Figures in the Text

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INTRODUCTION

Experimental procedure.

THE technique used for the present study of the interaction of potassium and light intensity was similar to that used for a previous study of the interaction of nitrogen and light intensity (White, 1937a) to which reference should be made. A strain of *Lemma minor*, originally obtained from the Isle of Wight and used for previous work (White 1936a, 1936b, 1936c, 1937a, 1937b), was not available and a strain from Hertfordshire was used, which was characterized, under corresponding conditions, by lower rates of multiplication and assimilation.

As in the previous experiment the plant colonies were grown in 30 c.c. Pyrex beakers and the nutrient solution replaced by fresh at intervals of

twelve hours. These beakers stood in sets of four within circular dishes, which were placed under four water-cooled gas-filled lamps, either of different wattage or suspended at different distances. Light intensity at the surface of the colonies was estimated by a Holophane lumeter. The dishes were placed in a water-bath with temperature maintained constant at 25° C. by heating lamps regulated by a mercury thermo-regulator. The treatments given consisted of the sixteen possible combinations of four light intensities and four potassium concentrations, the potassium level of the nutrient solution¹ being varied by altering the concentration of K₂SO₄. The levels of the factors used were as follows:²

Light intensity (continuous illumination): 300, 180, 120, and 60 ft.-candles.
Potassium concentration: 200, 20, 0.125, and 0.0 mg. potassium per litre.

Growth measures.

Area was estimated by drawing an enlarged outline of a sample focused on a ground-glass screen, and tracing with a planimeter, as described by Ashby, Bolas, and Henderson (1928), and dry weight by drying *in vacuo* under standard conditions and weighing on a microchemical balance, as described by Su and Ashby (1929). These samples consisted of eight plants of two fronds each. Notes were made of the relative depth of frond colour throughout the experiment. At the end of the experiment estimates of relative starch and protein content were made by plunging whole plants into hot water, decolorizing with warm alcohol, and staining with iodine and Millon's reagent. Where sufficient fronds remained after these tests had been carried out, estimates were made of the amylolytic activity of fresh samples calculated (from parallel samples used for dry weight determinations) to be of equivalent dry weight.

The relative rates of increase of the colonies (percentage increase per day of 24 hours continuous illumination) are estimated from differences between the natural logarithms of frond number, allowance being made for the random removal of plants to prevent overcrowding and of samples for area and weight determinations. If *FN* is the frond number, as estimated from frond count, *a* the average frond area of the sample, as estimated by tracing and planimeter, and *w* the average frond dry weight, as estimated by weighing, then *A* (the total area of the colony) = (*FN*) (*a*) and *W* (the total dry weight of the colony) = (*FN*) (*w*). If *a* and *w* remain constant, then change in rate of increase in area and dry weight of a colony is dependent on rate of increase in frond

¹ CaH₄(PO₄)₂ 0.101 gm.; MgSO₄ 0.255 gm.; Ca(NO₃)₂ 4 H₂O 0.116 gm.; Mg(NO₃)₂ 6 H₂O 0.077 gm.; Fe₂Cl₆ 0.002 gm.; K₂SO₄ 0.023 gm.; distilled water 1,000 c.c.

² The experiment was continued for twenty days, but the maximal potassium treatment of 200 mg. per litre was not begun until the fifth day. The range of potassium concentrations originally selected included one of 0.03 mg. per litre, but the preliminary response of the colonies in this solution suggested that they would differ but slightly from the colonies grown without potassium. This treatment was therefore discontinued and the colonies transferred to solutions with 200 mg. potassium per litre.

number, i.e. the rate of production of new meristems. The specific effect of treatment on area and dry weight of a colony must therefore be evaluated by the rate of change of a and w and not of A and W .

The dry weight per unit area of a colony, DW/A , is affected either by variation in frond area (through change in cell number or cell size) or in net assimilation rate. If the factors that influence cell number and cell size have relatively slight or a contrary effect on assimilation rate, then changes in frond area would be associated with inverse changes in dry weight per unit area.

If the net assimilation rate of a colony is estimated on a dry weight basis (increase in dry weight per unit of weight per unit time) then

$$N.A.R._w = \frac{W_2 - W_1 (\log_e(W_2/W_1))}{W_2 - W_1},$$

since the average weight of a colony between times t_1 and t_2 is

$$\frac{W_2 - W_1}{(\log_e W_2 - \log_e W_1)},$$

where W_1 and W_2 are the weights of the colony at times t_1 and t_2 respectively. Since W_2 and $W_1 = (FN_2)(w_2)$ and $(FN_1)(w_1)$ then $N.A.R._w = (w) \log_e FN_2/FN_1$, i.e. if (w) is constant, the value for net assimilation rate is dependent on the relative rate of increase in frond number. Similarly, if the net assimilation rate is calculated on an area basis (increase in dry weight per unit area per unit time), then $N.A.R._a = w/a \log_e FN_2/FN_1$, i.e. if frond dry weight is constant then the value for net assimilation rate is directly related to rate of increase in frond number and inversely related to rate of change in frond area.

The formula used to obtain the rate of net assimilation gives a valid estimate of the rate of increment of dry weight to the colony, but it must not be overlooked that this rate of increment is subject to the influence of the rate of formation of new meristems as well as to the rate of assimilation. This consideration does not arise only in the case of a *Lemna* colony in that all plants which are forming new meristems relatively rapidly increase in dry weight, other factors being equal, faster than plants which are forming new meristems relatively slowly, but it is of greater importance in the case of a *Lemna* colony since translocation and storage of carbohydrate in a *Lemna* plant is restricted, so that change in rate of production of new fronds and consequent demand for carbohydrates from the parent frond are reflected directly in the dry weight, as shown by previous findings (White, 1936c, p. 839; Dickson, 1938, p. 104) of a general inverse relationship between changes in multiplication rate and frond dry weight.

Although the desirability of replication for the purpose of statistical analysis of the results was recognized it was found imperative, in order to keep the work within the scope of a single investigator, to allot one colony only to each treatment. Under the rigidly controlled conditions of these experiments

and with colonies derived from the splitting of a single clone the variance that may be calculated from day-to-day fluctuations in growth-rate appears to exceed that estimated from differences between duplicate colonies (see Ashby and Oxley, 1935, Tables I and III), while that estimated from differences in dry weight and area between plants selected at random from different parts of the same colony is at least as great as that estimated from differences between replicate colonies (White, 1937a, p. 632). The use of these variances to estimate error of treatment would thus appear to underestimate rather than overestimate the significance of differences between treatments.

EXPERIMENTAL RESULTS

Frond number.

Increase in frond number of colonies with ample potassium supply was exponential throughout the experiment. Increase of colonies transferred to nutrient solutions lacking potassium was at first exponential but subsequently fell with time, and it is of interest to note that the period during which the exponential rate of increase was retained is prolonged by decrease in light intensity, as shown in Table I.

TABLE I
Duration of Period of Exponential Increase of Colonies Transferred to a Nutrient Solution with No Added Potassium

	300 ft.-candles	8 days
180	"	14 "
120	"	14 "
60	"	16 "

TABLE II

Relative Rates of Increase in Frond Number per Day (24 Hours Continuous Illumination) and Frond Numbers (brackets) of Colonies with all Combinations of Four Light Intensities and Three Potassium Levels

	Potassium (mg. per litre).		
	0·0.	0·125.	2·0.
300 ft.-candles	0·143 (1615)	0·159 (2240)	0·185 (3710)
180 "	0·134 (1360)	0·149 (1838)	0·166 (2540)
120 "	0·116 (964)	0·134 (1360)	0·140 (1542)
60 "	0·060 (320)	0·064 (349)	0·069 (386)

The relative rates of increase in frond number of the colonies are given in Table II, which shows that growth falls off with decrease in either light intensity or potassium supply at all levels of either factor. The values in brackets, which are the frond numbers to which a colony numbering 100 fronds on the first day of the experiment would increase after twenty days' growth, emphasize the magnitude of the cumulative effect of differences in relative growth rate. The relation between rate of increase in frond number and light intensity at three levels of potassium supply is plotted in Fig. 1. These curves of

growth at different potassium levels retain a proportionate relationship as the light intensity is lowered and show no tendency to intersect, contrasting in this respect with curves of growth at different nitrogen levels and the same range of light intensity (White 1937a). The trend of the curves suggests that the minimal light intensity that will support growth is of the order of 40–50 ft.-candles.

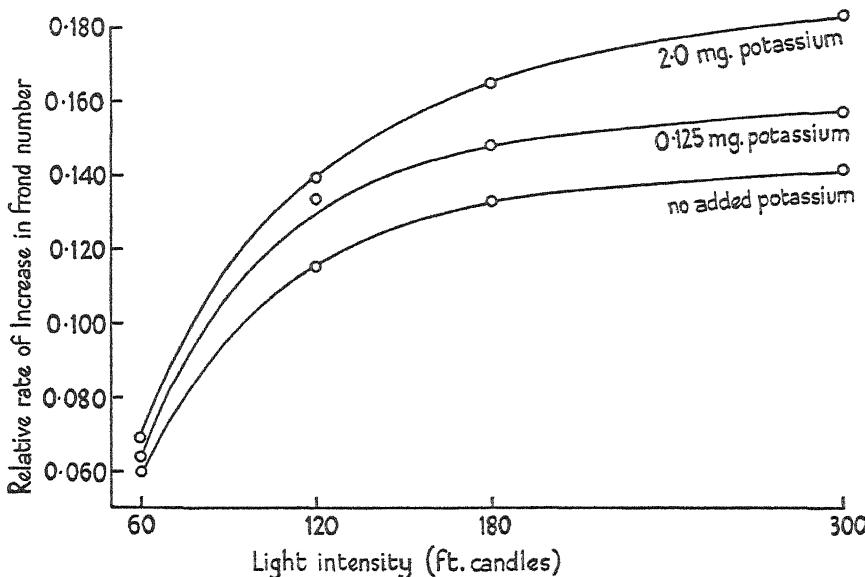


FIG. 1. Relative rate of increase in frond number plotted against light intensity for three levels of potassium supply.

TABLE III

Comparison of Relative Rates of Increase in Frond Number per Day (24 Hours Continuous Illumination) of Colonies with 200 and 2.0 mg. Potassium per Litre

	A (200 mg.).	B (2.0 mg.).	A/B.
300 ft.-candles	0.202	0.181	112%
180 "	0.169	0.163	104%
120 "	0.146	0.148	99%
60 "	0.064	0.068	94%

The rates of increase of the colonies in solutions with 200 mg. potassium per litre in comparison with those with 2.0 mg. potassium per litre are shown in Table III. The growth of the colony with higher potassium level is relatively faster under high light intensity and relatively slower under low light intensity.

Frond area.

Samples for estimation of area and dry weight were taken from each of the colonies under 300 and 180 ft.-candles at successive intervals of two days, making ten sampling occasions in all. The colonies under 120 ft.-candles,

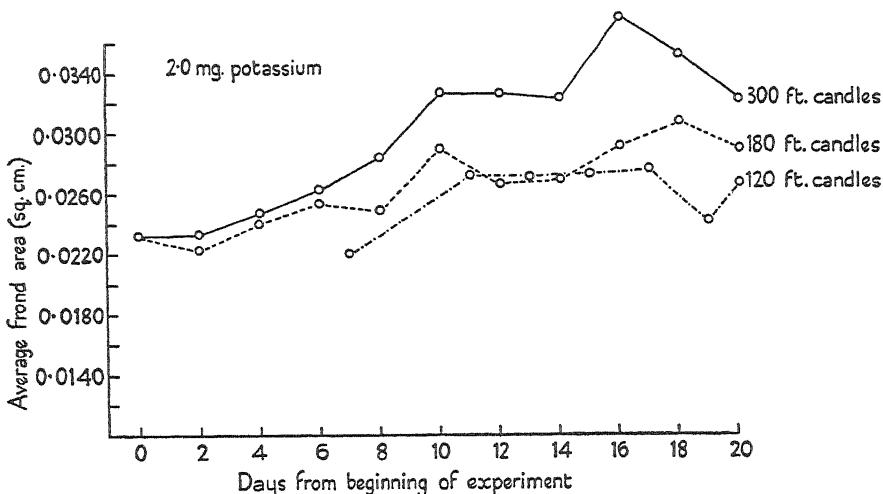


FIG. 2. Average frond area of colonies with a potassium supply of 2.0 mg. per litre at three levels of light intensity. (Cf. with Figs. 3 and 4.)

that increased in numbers more slowly, provided samples on seven occasions, while those under 60 ft.-candles could be sampled on two occasions only, as a sufficient number of fronds for estimating rate of increase in frond number would not otherwise have been available. The sampling of these colonies at 60 ft.-candles was, however, carried out in quadruplicate at the end of the experiment. Duplicate samples of the colonies under higher light intensities were taken at the end of the experiment and on one occasion during the experiment.

Fig. 2 shows the average frond area throughout the experiment of colonies grown with potassium concentration of 2.0 mg. per litre under three intensities of light. The highest light intensity of 300 ft.-candles has clearly the most favourable effect and the lowest light intensity of 120 ft.-candles the least favorable effect. Fig. 3 shows the result of reduction in potassium supply from 2.0 to 0.125 mg. per litre. For the greater part of the experiment there is now no detectable effect of variation in light intensity. Frond area falls away, however, under the highest light intensity of 300 ft.-candles during the last six days of the experiment. Fig. 4 shows frond area under the same light intensities but of colonies without potassium, i.e. with a further reduction in the level of the potassium factor. Over the first ten days of the experiment there is again no detectable effect of variation in light intensity, but during

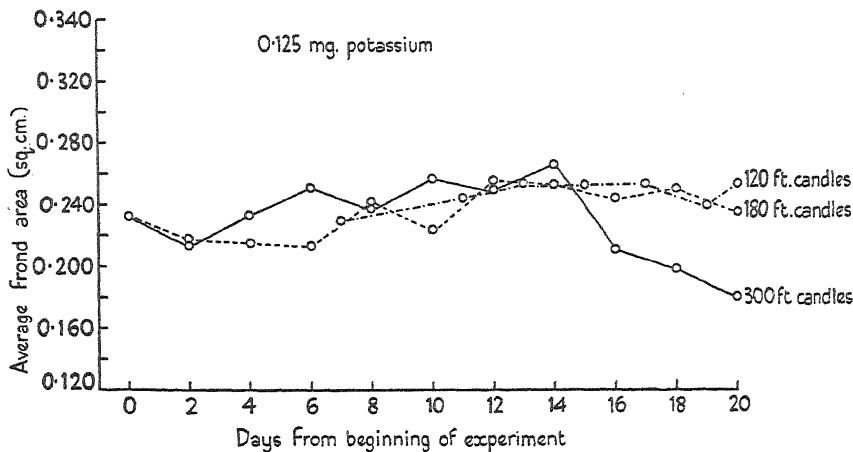


FIG. 3. Average frond area of colonies with potassium supply of 0.125 mg. per litre at three levels of light intensity. (Cf. with Figs. 2 and 4.)

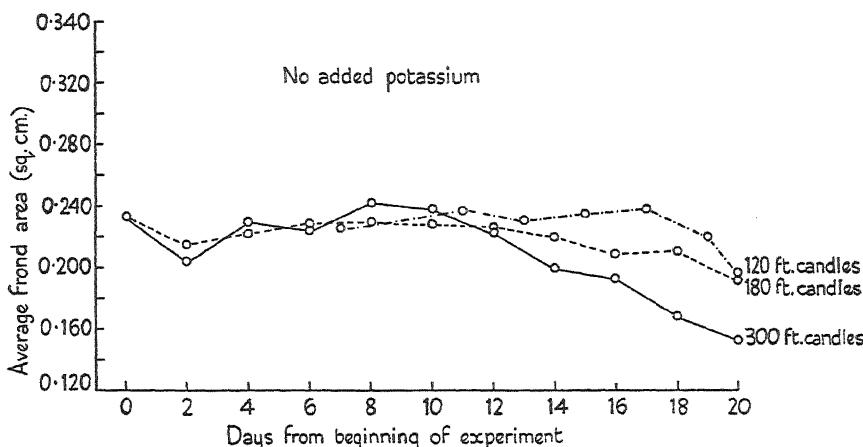


FIG. 4. Average frond area of colonies with no potassium added to the nutrient solution at three levels of light intensity. (Cf. with Figs. 2 and 3.)

the last ten days the lowest light intensity of 120 ft.-candles has the most favorable effect and the highest light intensity of 300 ft.-candles the least favorable effect. *The effect of variation in light intensity is thus completely reversed by reduction of potassium supply.*

It should be noted that all the colonies allotted to the different treatments on the first day of the experiment had been obtained by subdivision of a

large colony grown at 25° C. under continuous illumination of 300 ft.-candles for three weeks previously. Thus the initial frond area was the same with all treatments. Since each sample consisted of eight plants of two fronds each, the statistical significance of the differences between pairs of treatments for corresponding days may be estimated by Students' method of 't'. Table IV, which refers to the last three samplings of the experiment, shows that the differences claimed are highly significant.¹

TABLE IVa

Comparison of Average Frond Area (sq. cm.) of Colonies with 2·0 mg. Potassium per Litre under Light Intensities of 300 and 120 Ft.-candles

Number of sample.	300 ft.- candles.	120 ft.- candles.	$\bar{x} - \bar{y}$	n	t	P
8	0·0378	0·0277	0·0101	14	3·96	< 0·01
9	0·0353	0·0243	0·0110	14	4·83	< 0·01
10	0·0323	0·0268	0·0055	30	4·61	< 0·01

TABLE IVb

Comparison of Average Frond Area (sq. cm.) of Colonies without Potassium, under Light Intensities of 300 and 120 Ft.-candles

Number of sample.	300 ft.- candles.	120 ft.- candles.	$\bar{y} - \bar{x}$	n	t	P
8	0·0193	0·0239	0·0046	14	5·30	< 0·01
9	0·0168	0·0220	0·0052	14	5·89	< 0·01
10	0·0153	0·0196	0·0043	30	6·20	< 0·01

TABLE V

Average Frond Area (sq. cm.) of Colonies with All Combinations of Four Light Intensities and Four Potassium Levels

	Potassium (mg. per litre).			
	0·0.	0·125.	2·0.	200.
300 ft.-candles	0·0171	0·0197	0·0351	0·0355
180	,,	0·0204	0·0244	0·0298
120	,,	0·0218	0·0249	0·0263
60	,,	0·0185	0·0167	0·0184
				0·0178

¹ The validity of the assumption that these differences are due to the treatments applied rests upon the use of variance within colonies to estimate error of treatment in place of variance between colonies, which is not available owing to lack of replication. The justification for this procedure has been discussed in a previous communication (White, 1937a, p. 632) to which reference should be made.

The relative effect of the sixteen treatments studied is seen in Table V, which gives the means of the samples taken during the last six days of the experiment. For the two lower potassium levels maximal frond area is obtained with 120 ft.-candles, whereas for the two higher potassium levels

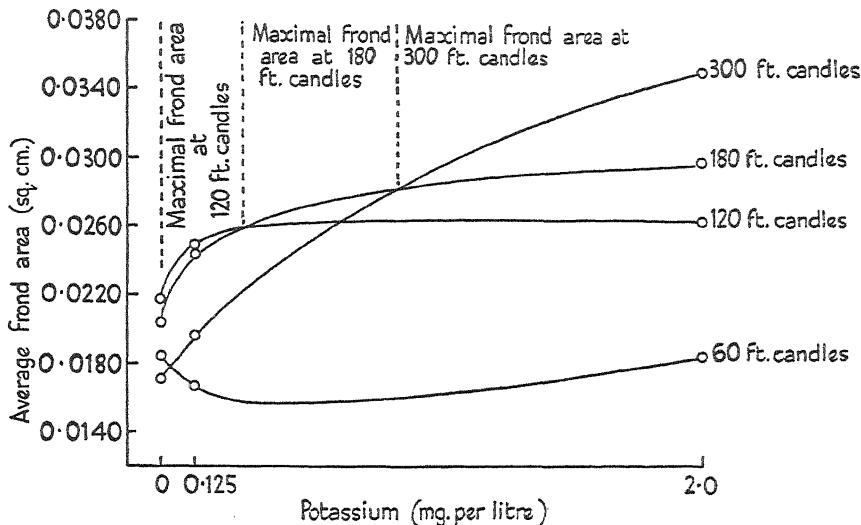


FIG. 5. Average frond area plotted against potassium supply for four levels of light intensity. Attention is directed to the shift of optimal light intensity to lower levels as the potassium supply is reduced by the insertion of arbitrary divisions corresponding with the light intensities used. From the trend of the curves it is evident that the intersecting points must approximate to the positions indicated.

maximal frond area is obtained with 300 ft.-candles. The effect of variation in light intensity and potassium supply is shown graphically in Figs. 5 and 6. From the trend of the curves of Fig. 5 it is evident that the optimal light intensity for potassium concentrations between 2.0 and 1.0 mg. per litre must be of the order of 300 ft.-candles, between 1.0 and 0.25 mg. per litre of the order of 180 ft.-candles and below 0.25 mg. per litre of the order of 120 ft.-candles. Clearly with rising potassium supply the optimal light intensity rises and with falling potassium supply the optimal light intensity falls. The complementary effect of shift of optimal potassium supply with variation of light intensity is much less pronounced (Fig. 6). The difference of 10 per cent. between the colonies without potassium and with 0.125 mg. per litre at the minimal light intensity of 60 ft.-candles was, however, consistently obtained for each of the five samples covering the two occasions on which the frond area of these colonies was estimated, and this suggests that the potassium curves intersect at very low light intensities.

Fig. 6 shows that with rise in light intensity from 60 to 120 ft.-candles there is an increase in frond area at all potassium levels. With further increase

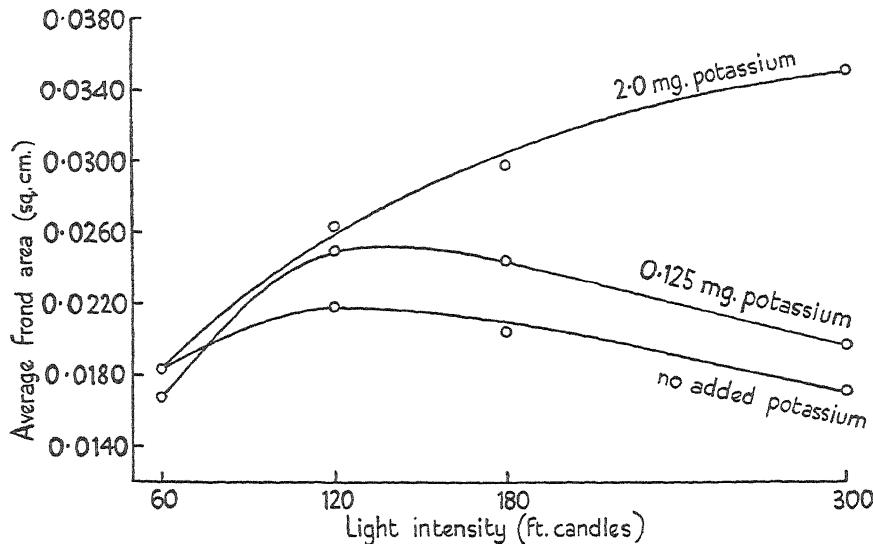


FIG. 6. Average frond area plotted against light intensity for three levels of potassium supply.

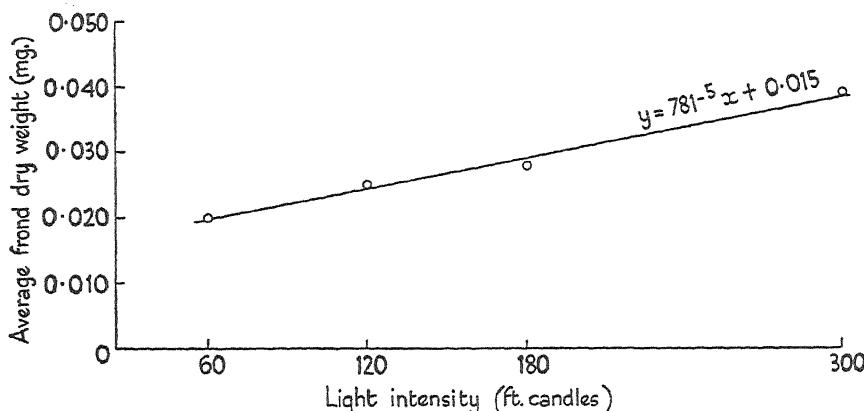


FIG. 7. Frond dry weight against light intensity. The values are the means of all potassium treatments.

to 300 ft.-candles there is an increase in frond area with high potassium supply (2.0 mg.) but an appreciable decrease with low potassium supply (0.125 and 0.0 mg.). This results in the curves relating frond area and light intensity having well-marked optima with low potassium level whereas with high potassium level the curve rises over the whole range of light intensity. It is evident that with intermediate potassium level the curve must flatten out so that there would be no change in frond area between 180 and 300 ft.-candles.

Frond dry weight.

At the highest light intensity of 300 ft.-candles the average frond dry weights during the last six days of the experiment are 0.041 and 0.043 mg. for the colonies with potassium treatments of 2.0 and 0.125 mg. per litre and 0.035 and 0.037 mg. for the colonies with 0.125 and 0.0 mg. per litre. At the lowest light intensity of 60 ft.-candles this trend is reversed, as may be seen from a comparison of the average frond dry weights of four samples taken from the colony with 2.0 mg. potassium per litre at the end of the experiment (0.019, 0.017, 0.018, and 0.018 mg.) with corresponding values for the colony without potassium (0.022, 0.023, 0.021, and 0.020 mg.). The mean values for all potassium treatments are shown in Fig. 7, which illustrates that there is a direct relation between frond dry weight and light intensity.

TABLE VI

Dry Weight per Unit Area (mg. per sq. cm.) of Colonies with all Combinations of Four Potassium Levels and Four Light Intensities

Potassium (mg. per litre).				
300 ft.-candles	0.0.	0.125.	2.0.	200.
180	2.02	1.62	1.25	1.20
120	1.30	1.12	0.98	1.09
60	1.23	0.99	0.95	0.96
	1.10	1.06	1.04	1.07

Dry weight per unit area.

The mean dry weight per unit area of samples taken during the last six days of the experiment, when treatment effects were maximal, is shown in Table VI. The finding of a previous communication (White, 1936a) that high dry weight per unit area is associated with potassium starvation is confirmed by Table VI, which shows also that the magnitude of this effect is progressively reduced by decrease of light intensity. Further, it is evident that the direct relationship previously recorded between dry weight per unit area and light intensities of from 1,200 to 300 ft.-candles (White, 1936c, Fig. 5) is not maintained with further reduction from 300 to 60 ft.-candles, *colonies under light intensities lower than 180 ft.-candles having almost the same dry weight per unit area*. Fig. 8, giving dry weight per unit area plotted against light intensity for three levels of potassium supply, shows that the dry weight per unit area of colonies with 0.125 and 2.0 mg. potassium per litre falls to a *minimum* at about 130 ft.-candles, and that further reduction in light intensity is associated with a *rise* in dry weight per unit area.

Net assimilation rate.

Net increase in dry weight per unit area in unit time has been estimated by

$$\frac{(W_2 - W_1)(\log_e A_2 - \log_e A_1)}{A_2 - A_1},$$

where A_1 , A_2 , W_1 , W_2 are the total area and total dry weight of the colonies at times t_1 and t_2 respectively. The mean net assimilation rates on an area basis of the colonies throughout the experiment are given in Table VII, which shows that with light intensities of from 300 to 120 ft.-candles decrease of potassium supply is associated with fall in net assimilation rate, although the magnitude of this decrease is not appreciable below the 2·0 mg. potassium level. This, however, is the result of sharp rises in net assimilation rate immediately following transference to potassium-free solutions, the starved colony under 300 ft.-candles registering a 17 per cent. increase over the colony with 2·0 mg. potassium per litre during the first four days of starvation in contrast with a 35 per cent. decrease at the end of the experiment.

TABLE VII

Net Assimilation Rates (mg. per sq. cm. per two days) of Colonies with all Combinations of Three Light Intensities and Four Potassium Concentrations. Corresponding Values calculated on a Dry Weight Basis (mg. per mg. dry weight per two days) are given in brackets

	300 ft.-candles	Potassium (mg. per litre).			
		0·0.	0·125.	2·0.	200.
	0·48 (0·28)	0·48 (0·31)	0·51 (0·38)	0·55 (0·43)	
180	"	0·31 (0·24)	0·31 (0·26)	0·33 (0·30)	0·36 (0·35)
120	"	0·18 (0·13)	0·19 (0·15)	0·19 (0·27)	0·23 (0·28)
60	"	0·15 (0·13)	0·13 (0·12)	0·09 (0·08)	0·08 (0·07)

Owing to the rate of growth under a light intensity of 60 ft.-candles being insufficient to balance the number of fronds required for sampling, area and dry weight data were not obtained during the early part of the experiment and the values for net assimilation rate at this light intensity refer, therefore, to the last eight days of the experiment only. Table VII shows that at this light intensity net assimilation rate rises with decreasing potassium supply so that *the maximal net assimilation rate is that of the colony with zero potassium supply*.

Values for net assimilation rate calculated on a dry weight basis (given in brackets in Table VII) show similar trends to those calculated on an area basis, but the fall associated with decrease in potassium supply is accentuated whereas that associated with decrease in light intensity is less pronounced. The relation between net assimilation rate and light intensity at different levels of potassium supply is plotted in Fig. 9. *The effect of variation in potassium supply on net assimilation rate at low light intensity is the reverse of that at high light intensity.*

Starch content, protein content, depth of frond colour, and amyloytic activity.

Samples of fronds from each treatment were plunged in hot water, de-colorized in warm alcohol, and stained under standard conditions with iodine and Millon's reagent. Notes were made on depth of frond colour, and the

fronds remaining at the end of the experiment were used for an estimate of amylolytic activity by a method previously described (White, 1936a, p. 190). The latter results do not include the lowest light intensity (60 ft.-candles)

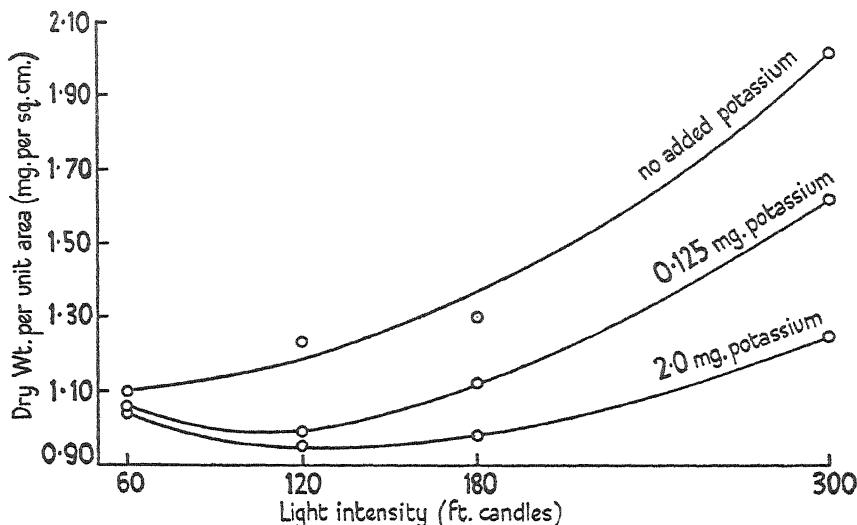


FIG. 8. Dry weight per unit area plotted against light intensity for three levels of potassium supply.

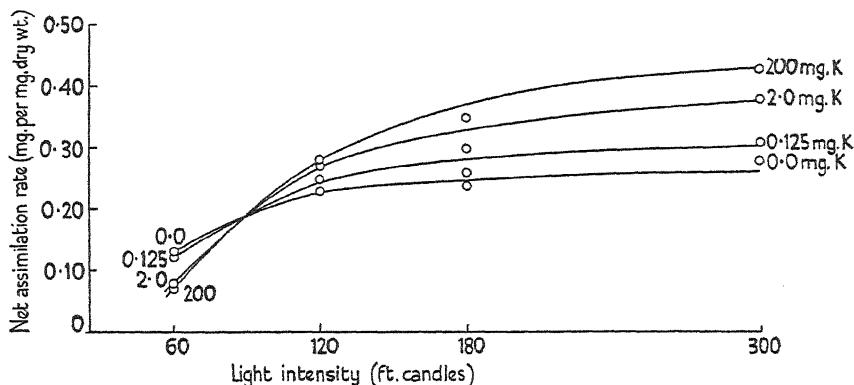


FIG. 9. Net assimilation rate on a dry weight basis plotted against light intensity for four levels of potassium supply. The values plotted cover the period of the last six days of the experiment.

since all the fronds from these colonies had already been used for other tests. The starch, protein, and frond colour results, expressed on arbitrary scales of 0 (no starch, dense protein, frond colour very pallid) to 10 (dense starch, dense protein, deep frond colour) are represented graphically in Figs. 10-12.

Fig. 10 confirms the previous finding (White, 1936a) that potassium starvation is associated with high starch content, and shows also that the magnitude of this starch increase is progressively reduced by decrease of light intensity from 300 to 60 ft.-candles. Fronds from every colony contained starch, but the distribution of starch in fronds under the lowest light intensity of 60 ft.-candles was somewhat irregular, some fronds being colourless and others blackened by iodine. Whereas the starch content of colonies with 200 and 2·0 mg. potassium per litre falls with decrease of light intensity from 180 to 60 ft.-candles, dry weight per unit area does not (Table VI). This suggests that the starch-sugar ratio falls with decrease in light intensity from 180 to 60 ft.-candles. The results of determinations of the relative rates of hydrolysis of a standard starch solution by ground samples, given in Table VIII, are in agreement with this view, for the amylolytic activity of colonies with 200 and 2·0 mg. per litre increases as the light intensity is reduced from 300 to 120 ft. candles.¹

TABLE VIII

Time (hours) Taken by Samples of Equivalent Dry Weight to Hydrolyse a given Starch Solution

		Potassium (mg. per litre).							
		0·0.		0·125.		2·0.		200.	
		28·0	29·0	18·0	25·0	9·0	13·5	4·5	4·0
300 ft.-candles	30·0			28·0		18·0		3·5	
	,,	2·0	2·5	2·0	2·25	2·0	2·25	2·0	2·25
180	3·0			2·5		2·5		2·5	
	,,	3·5	3·0	2·0	2·75	1·0	1·0	1·0	1·25
120	2·5			3·5		1·0		1·5	

Fig. 11 shows that decreasing potassium supply under a light intensity of 300 ft.-candles is associated with falling protein content. At lower light intensities differences in protein content are not sufficiently marked to be considered definite. Fig. 11 shows further that decreasing light intensity has a detrimental effect on the protein content of the colonies with a high potassium supply in contrast to a favourable effect of decreasing light intensity on the protein content of the colonies without potassium.

Fig. 12 shows that under the highest light intensity of 300 ft.-candles frond

¹ The values corresponding with decrease of potassium supply at a light intensity of 300 ft.-candles have been considered in a previous communication (White, 1936a). In recording the method by which these tests were carried out it was not stated that these values are subject to a minor correction in that the fronds of the colonies without potassium contain surplus starch, which is included in the test. If it be assumed that the difference in dry weight per unit area of 1·12 mg. per sq. cm. between the colony with 200 mg. per litre and the colony without potassium consists entirely of starch, then the corresponding addition, in a sample of 25 fronds, of about 0·6 mg. starch to the 0·2 c.c. of a 2 per cent. starch solution used as a standard, represents an increase of about 15 per cent. and is equivalent to a similar increase in the time required for hydrolysis. Since the difference recorded in Table VIII is over 700 per cent., this correction does not affect the conclusion that potassium starvation is associated with low amylolytic activity.

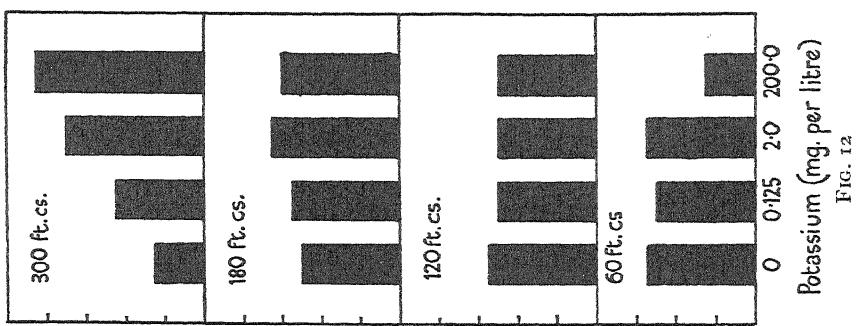


FIG. 12

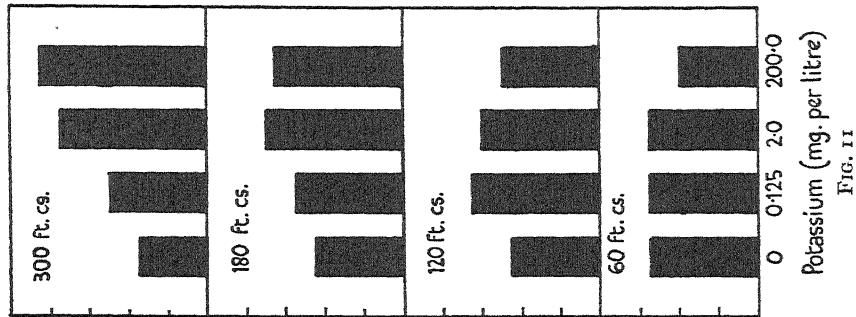


FIG. 11

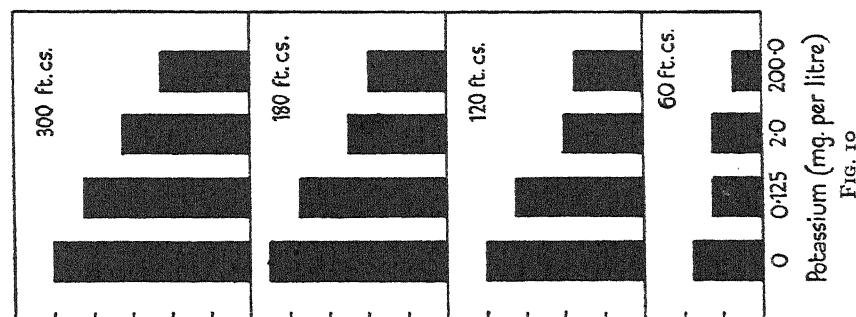


FIG. 10

Figs. 10-12. Fig. 10. The heights of the columns give a measure of the relative starch content, estimated from the intensity of iodine stain, of colonies subjected to sixteen combinations of light intensity and potassium supply. Fig. 11. The heights of the columns give a measure of the relative protein content, estimated from the intensity of stain with Millon's reagent, of colonies subjected to sixteen combinations of light intensity and potassium supply. Fig. 12. The heights of the columns give a measure of the relative depth of frond colour of colonies subjected to sixteen combinations of light intensity and potassium supply.

colour becomes paler with decreasing potassium supply, but under the lowest light intensity of 60 ft.-candles the colour of the colony with the highest potassium level is paler than with lower potassium supply. With the highest potassium level of 200 mg. per litre the depth of frond colour falls off with decreasing light intensity, but without potassium the colony under the lowest light intensity of 60 ft.-candles has fronds of deeper colour than those of the colony under the highest light intensity of 300 ft.-candles. *The effect of variation of either factor is reversed by a change from a high level to a low level of the other factor.*

The values shown in Fig. 12 refer, however, only to the conditions at the end of the experiment. Careful notes made during the experiment show that under 300 ft.-candles the colony without potassium, before becoming paler in colour than the colonies with higher potassium supply, passed through a stage (9th and 12th days of experiment) in which it was definitely darker. The corresponding colony under 180 ft.-candles behaved in a similar manner later in the experiment, while the colony under 120 ft.-candles only appeared darker than the colonies with higher potassium supply just before the end of the experiment.

The trends of starch content, protein content, and depth of frond colour with variation in potassium supply at different light intensities resemble closely corresponding trends with variation in nitrogen supply under a similar range of light intensities (White, 1937a, Figs. 9-11). Differences in frond colour were, however, relatively clear cut and differences in protein content less marked with variation in potassium supply, whereas with variation in nitrogen supply differences in protein content were relatively clear-cut and differences in frond colour less marked.

DISCUSSION

Correlation between measures of growth.

Correlation coefficients between different growth measures under the sixteen combinations of light intensity and potassium supply are assembled in Table IX. In order that all the growth measures referred to should represent the maximal effect of treatment the values for rates of frond multiplication and net assimilation are those for the last six days of the experiment.

Rate of increase in frond number is significantly correlated with net assimilation rate (see p. 621), frond area, protein content, and depth of frond colour. The partial correlation coefficient between rate of increase in frond number and protein content, eliminating net assimilation rate (+0.496), is significant. Protein content is also significantly correlated with (1) frond area, (2) net assimilation rate, (3) depth of frond colour. With reference to the latter result it is of interest to note the finding of Gassner and Goeze (1932) that there is an association between leaf colour and protein content in the leaves of wheat seedlings.

TABLE IX

Correlation between Growth Measures of Colonies with Sixteen Combinations of Light Intensity and Potassium Supply

Significant values in heavy type

Frond area	+ 0.822				
Net assimilation rate	+ 0.882	+ 0.237			
Dry weight per unit area	+ 0.058	- 0.158	+ 0.416		
Starch	+ 0.321	- 0.042	+ 0.481	+ 0.810	
Protein	+ 0.653	+ 0.896	+ 0.496	- 0.462	- 0.242
Depth of frond colour	+ 0.567	+ 0.308	+ 0.477	- 0.353	- 0.114
					+ 0.888
	Rate of increase in frond number	Frond area	Net assimilation rate	Dry weight per unit area	Starch
					Protein

The negative correlation between dry weight per unit area and frond area is not significant. This correlation, in which are combined the effects of both potassium and light treatments, may be compared with the striking negative correlation ($r = -0.944$) between fluctuations in frond area and dry weight per unit area that was associated with variation in potassium supply alone in a previous experiment (White, 1936a). In order to separate the effects of variation in potassium supply and light intensity in the present experiment the coefficients between the values of area and dry weight per unit area corresponding with different potassium treatments have been calculated for each light intensity separately. The results, shown in Table X, confirm the previous experiment, striking negative correlations being associated with variation in potassium treatment at the two higher light intensities. As the light intensity is reduced the magnitude of the differences between the colonies growing in different potassium concentrations decreases and the correlation between frond area and dry weight per unit area disappears, the coefficient ceasing to be significant at 120 ft.-candles and being converted to a moderate positive value at 60 ft.-candles. It appears that an inverse relationship between frond area and dry weight per unit area is occasioned by variation in potassium supply but not by variation in light intensity. Further evidence is furnished by the fluctuations from their mean values of frond area and dry weight per unit area throughout the experiment in the different treatments. These correlation coefficients, which are invariably negative, are assembled in Table XI. The significance of the values for the colonies without potassium

or with low potassium supply contrasts with the lack of significance of the values for the colonies with 200 mg. potassium per litre and emphasizes the dependence on potassium level of these inverse fluctuations of area and dry weight. Starch content is closely correlated ($r = +0.810$) with dry weight per unit area in Table IX and it is evident that with fluctuation in potassium supply there must be an inverse relationship also between starch content and frond area.

TABLE X

The Effect of Light Intensity on the Correlation between Values of Frond Area and Dry Weight per Unit Area of Colonies with Different Potassium Treatments

Significant values in heavy type	
Light intensity. (ft.-candles)	<i>r.</i>
300	-0.955
180	-0.974
120	-0.882
60	+0.484

TABLE XI

Correlation Coefficients between Fluctuations in Average Frond Area and Dry Weight per Unit Area from the Mean Values, during Twenty Days' Growth, of Colonies with Four Potassium Levels under Three Light Intensities. The Number of Occasions of Sampling Range from Seven to Ten

Potassium (mg. per litre).	Light intensity (ft.-candles).		
	300	180	120
200	-0.644	-0.479	-0.469
2.0	-0.621	-0.835	-0.890
0.125	-0.883	-0.772	-0.920
0.0	-0.893	-0.688	-0.934

The carbohydrate metabolism of potassium-starved colonies.

The influence of potassium on carbohydrate metabolism has been considered in a previous communication (White, 1936a) in which a twofold effect of potassium starvation was indicated: (1) low assimilation rate, (2) high starch content and low amyloytic activity.

The effect of potassium in the present experiment may be assessed from a detailed comparison of the colony without potassium at 300 ft.-candles with the colony with 2.0 mg. potassium per litre. Relative values in these two colonies for multiplication rate, frond area, frond dry weight, dry weight per unit area, root length and net assimilation rate, calculated on both area and dry weight basis, are plotted in Figs. 13 and 14, the values for the colony receiving 2 mg. potassium per litre being the standard of reference. As an aid to the interpretation of the results linear regression lines have been fitted

to the data (from the 2nd to the 18th day of the experiment) and regression coefficients, representing mean percentage changes per day, are shown in Table XII.

TABLE XII

*Percentage Change per Day of a Colony without Potassium Relative
to a Colony with 2 mg. Potassium per Litre*

Rate of increase in frond number	-2·1
Average frond area	-2·8
Average frond dry weight	-1·4
Net assimilation rate (calculated on an area basis) (calculated on a dry-weight basis)	-3·1
Dry weight per unit area	-4·1
Root length	+3·8
	-4·1

The curve of rate of increase in frond number (Fig. 13) falls less steeply (2·1 per cent. per day) than the curves of frond area (2·8 per cent. per day) and root length (4·1 per cent. per day). It is evident that the development of new meristems is affected less severely by potassium starvation than some of the other growth measures considered. Net assimilation rate calculated on a dry weight basis (Fig. 14) falls steadily throughout the experiment (4·1 per cent. per day). Net assimilation rate calculated on an area basis (Fig. 14) also falls throughout the experiment (3·1 per cent. per day), following a preliminary rise during the first four days. This rise is partly accounted for, however, by the fall in frond area, which tends automatically to raise the value of assimilation rate calculated on an area basis.

An outstanding feature of Fig. 13 is the steady fall throughout the experiment of frond area (2·8 per cent. per day) and rise in dry weight per unit area (3·8 per cent. per day), a further illustration of the inverse relationship between these growth measures associated with changing potassium level. It is noteworthy that this fall in frond area, apparent as early as the second day after transference of the colony to a nutrient solution without potassium, precedes the falls in rate of assimilation and increase in frond number that are later to be occasioned by potassium starvation. Since change in frond dry weight at this period is relatively slight it is evident that *potassium starvation has led to a reduction in water content*. Change in water-content of the potassium-starved frond clearly accounts for the inverse relationship between frond area and dry weight per unit area to which attention has been directed in the section on correlation between growth measures.

A decrease in frond area arising through reduction in water content should be associated with an increase of corresponding magnitude in dry weight per unit area. The increase in dry weight per unit area (3·8 per cent. per day) of the potassium-starved colony exceeds the corresponding decrease in frond area (2·8 per cent. per day) by 1 per cent. per day and this difference represents real carbohydrate accumulation, comprising increase in thickness of cell wall and of specific gravity of cell-contents, probably mainly increase of

starch (see Fig. 10). Increase in dry weight per unit area, after differences in frond area have been allowed for, measures the accumulation of carbohydrate over and above the minimal level requisite to maintain the growth-rate of the colony. Since the relative growth rate (rate of frond multiplication) falls

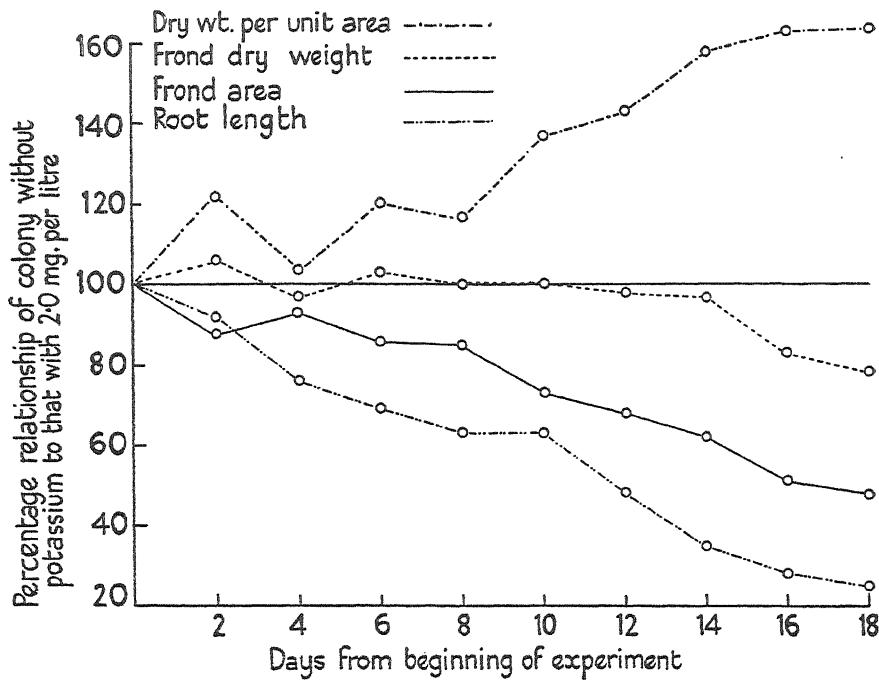


FIG. 13. Percentage relationship in frond area, dry weight and root length of a colony without potassium to a colony with potassium supply of 2·0 mg. per litre. The light intensity is 300 ft.-candles.

by 2·1 per cent. per day a rise of corresponding magnitude in the level of accumulated carbohydrate would be expected. The increase recorded is only 1 per cent. and it appears therefore that the *net assimilation rate per unit area must have been falling at a rate of about 1·1 per cent. per day*. An apparent discrepancy arises here between this value and that recorded in Table XII for net assimilation rate calculated as increment in dry weight divided by average frond area in unit time. The estimates of increment in dry weight by the latter method of calculation are subject, however, to the effects of changing rate of increase in frond number, which are reflected in the curves of net assimilation rate in Fig. 14. The effects of factors other than rate of increase in frond number may be estimated from the trend of differences between the curves of rate of increase in frond number and assimilation rate. The curve of net assimilation rate on dry weight basis rises to a level 9 per cent. above that of rate of increase in frond number during the first four days

of the experiment, and this suggests that potassium deficiency is associated at first with a *rise* in assimilation rate. Subsequently the curve for net assimilation rate (dry weight basis) falls below that of rate of increase in frond number, showing that a more severe stage of potassium starvation is associated with a *fall* in assimilation rate.

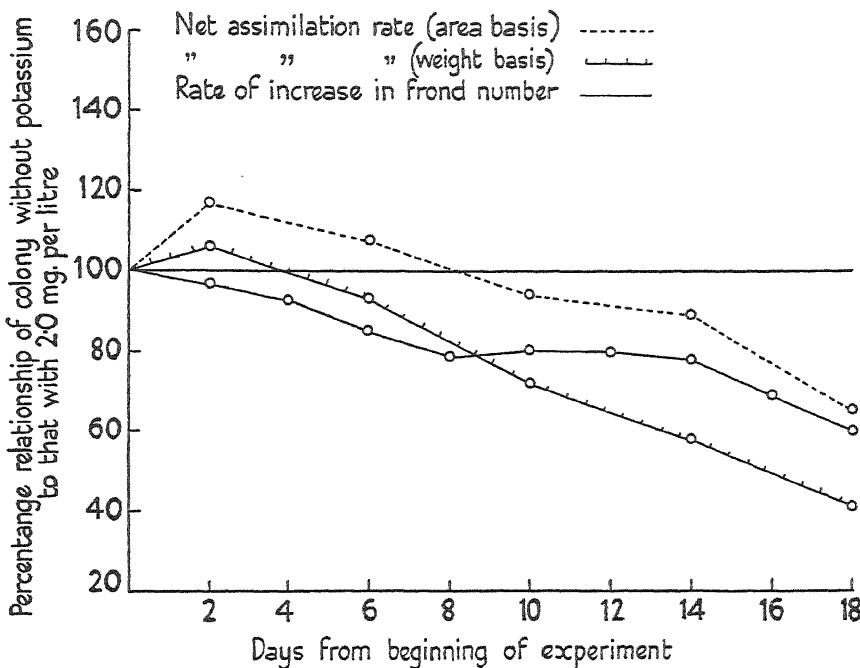


FIG. 14. Percentage relationship in rate of increase in frond number and net assimilation rate, referred to both area and dry weight bases, of a colony without potassium to a colony with 20 mg. per litre. The light intensity is 300 ft.-candles.

Since a fall in frond area has the effect of raising the value for net assimilation rate calculated on an area basis, the difference between rates of fall in net assimilation rate on area and weight bases is a measure of the extent to which frond area is affected by factors other than rates of assimilation and increase in frond number. This difference, amounting to 1 per cent. per day (Table XII), represents the extent to which potassium influences frond area through reduction in water content. Although data of cell size are not available, the detrimental influence of potassium starvation on water content may well be attributed to a reduction in cell size for it is a reasonable assumption that the *number* of cells are determined in the frond primordium and the rate of development of the frond primordium is relatively only slightly affected by potassium starvation (see p. 637). It is of interest to point out that the falling sugar level which may be presumed to be associated with rising starch

level (Fig. 10) and low amylolytic activity (Table VIII) might be expected to influence appreciably the suction pressure of the cells leading to reduction in cell size. In support of this view may be quoted the work of Warne (1936) who has shown that in the leaves of potassium-starved seakale beet reduction in cell-size is associated with a proportionate reduction in leaf-area (the number of cells appearing to be unchanged). The reduction in root length of *Lemna* (Fig. 13), immediately following omission of potassium from the nutrient solution, is also conformable with this view since it has been concluded that change in sugar concentration has a marked influence on root length (White, 1937*b*, 1938).

It is of interest to note that the effects of potassium starvation recorded in the present investigations of the metabolism of *Lemna* colonies are in general conformity with those recorded for other plants. Potassium deficiency has been associated with low assimilation rate in barley by Gregory and Richards (1929) and Richards (1932), in wheat by Gassner and Goeze (1932), and in *Fontinalis* and *Chlorella* by Pirson (1937) (1938). Increase in dry matter per unit area and decrease in water content has been reported by Janssen and Bartholomew (1930) and other investigators for a number of potassium-starved plants. The association of potassium starvation with starch accumulation is noted in soybean by Burrell (1926), in tomato, beet, carrot, parsnip, radish, &c., by Nightingale, Schemerhorn, and Robbins (1930), and in other plants by a number of other investigators. Starch increase at the expense of sugar concentration may be seen in the tables of Janssen and Bartholomew (1930).

Divergent conclusions have been reached in previous investigations of the influence of potassium on the activity of amylase. The decrease in amylolytic activity associated with potassium deficiency in the present series of experiments with *Lemna* is in agreement with a similar finding by Cattle (1933) for broad bean, while this effect is also indicated by the work of Englis and Lunt (1925) with nasturtium. On the other hand, Doby and Hibbard (1927) and Hartt (1929) (1934), who worked with sugar beet, associate potassium deficiency with the contrary effect of increased activity of amylase. Prevalent views as to the constitution and activity of enzymes suggest that the activity of amylase should be affected by changes in protein level (see White, 1926*b*, p. 415), and the divergent effects of potassium deficiency on amylolytic activity may therefore be a reflection of divergent changes in nitrogen metabolism.

The protein metabolism of potassium-starved colonies.

Significant correlation ($+0.888$) between frond colour and protein content has been recorded in Table IX, and this association is confirmed without exception (e.g. White, 1937*a*, p. 640) in other experiments with varying levels of light intensity, nitrogen supply, and potassium supply. Similar results have been recorded by other workers, e.g. Michael (1935). Although protein

level was only estimated at the end of the present experiment, frond colour was estimated every alternate day, and it is therefore of interest to assess the probable course of protein level throughout the experiment from the trend of frond colour, which, in the potassium-starved colony under 300 ft.-candles,

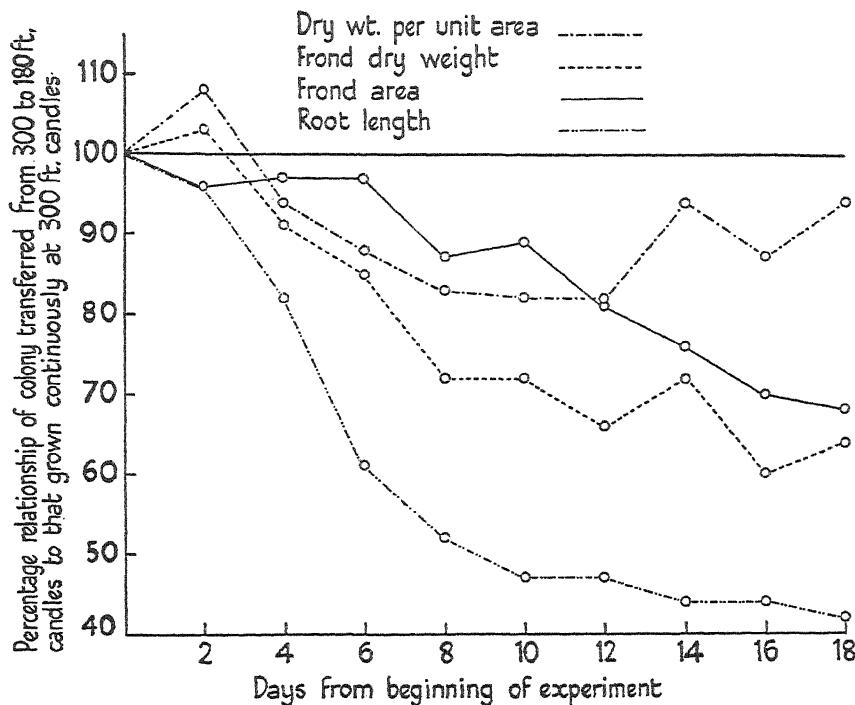


FIG. 15. Percentage relationship in frond area, dry weight, and root length of a colony under 180 ft.-candles to a colony under 300 ft.-candles. The levels of potassium supply are 2·0 mg. per litre (day 0-12) and 2·0 mg. per litre (day 12-18).

rose to maximal intensity about the tenth day of the experiment (see p. 634) and subsequently fell (Fig. 12). These considerations suggest that potassium starvation leads at first to an increase in protein level, despite falling rates of increase in frond number, area and dry weight, but ultimately to a decrease in protein level. The changes in protein level of colonies at lower light intensities at the end of the experiment support this view (Fig. 11).

The metabolism of colonies under low light intensity.

Since the colonies under 120 and 60 ft.-candles did not grow sufficiently rapidly to provide a number of successive samples, the progressive effect of change in light intensity must be assessed from the lesser differences between the colonies under 300 and 180 ft.-candles. These changes are shown in Figs. 15 and 16, the colony under 300 ft.-candles being used as a standard

of reference. The colonies were growing prior to the experiment under a light intensity of 300 ft.-candles. Figs. 15-16 record, therefore, the effect of transference of a colony from a higher to a lower light intensity. The immediate effect is a pronounced fall in rate of increase in frond number, in frond

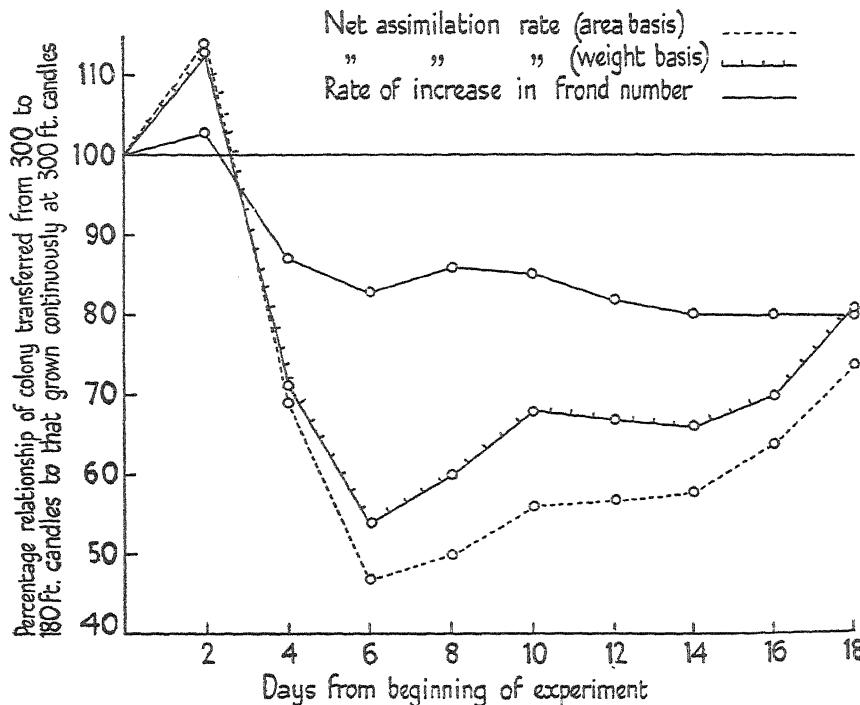


FIG. 16. Percentage relationship in rate of increase in frond number and net assimilation rate referred to both area and dry weight bases of a colony under 180 ft.-candles to a colony under 300 ft.-candles. The levels of potassium supply are 2·0 mg. per litre (day 0-12) and 200 mg. per litre (day 12-18).

dry weight, and in root length. Net assimilation rate falls steeply on both area and dry weight bases to values 50 per cent. below the former levels. Average frond area also falls steadily though less steeply than the frond dry weight. Subsequently the curves for rate of increase in frond number and frond dry weight, together with root length, show a tendency to flatten out at new levels and this results in appreciable recovery in the values for dry weight per unit area and net assimilation rate. According to the levels of adjustment indicated by Figs. 15 and 16, reduction in light intensity from 300 to 180 ft.-candles results in a fall in relative rate of increase in frond number of 20 per cent., in root length of 60 per cent., and in frond dry weight of 35 per cent. Frond area appears to be falling towards the same level as frond dry weight so that ultimately dry weight per unit area would rise to the same value as that of the

colony at the higher light intensity. It is of interest to note that the minimal period required for adjustment of the metabolism of the colony to a difference in light intensity of only 120 ft.-candles is at least twenty days.

A striking feature of decrease in light intensity is the differential effect demonstrated on rate of net assimilation (Fig. 9) and dry weight per unit area (Fig. 8). With ample potassium supply, reduction in light intensity from 300 to 180 ft.-candles is associated with a fall in net assimilation rate (area basis) of 35 per cent. (Table VII), while the corresponding fall in dry weight per unit area is 9 per cent. (Table VI). With further reduction in light intensity to 60 ft.-candles this difference is accentuated, for net assimilation rate falls by 85 per cent. (area basis) and 84 per cent. (weight basis), while dry weight per unit area differs only by 11 per cent. from that of the colony under 300 ft.-candles. Moreover, dry weight per unit area passes through a 'minimum' at about 130 ft.-candles and rises with further decrease in light intensity (Fig. 12). Evidently at low light intensities there is carbohydrate accumulation over and above that requisite for growth, which is not closely related to carbohydrate level. These considerations are difficult to reconcile with the conception of cessation of growth at low light intensity owing to the balancing of the carbohydrate gained through assimilation by that lost through respiration. Under the experimental conditions of continuous illumination of low intensity there is no evidence of a 'compensation point'. On the other hand, there is a marked fall in protein level with decrease in light intensity from 300 to 60 ft.-candles (Fig. 11). Either this is a direct effect of falling light intensity, a view that falls into line with the demonstration by Eckerson (1932) of retardation of reductase activity by decrease of light intensity or length of day, or an indirect effect through relative excess of potassium, a supposition that is not supported by the relatively slight increase¹ in protein level associated with decrease of potassium supply at 60 ft.-candles (Fig. 11). It is of considerable interest to note that the optimal nitrate concentration for protein content is markedly influenced by variation in light intensity, the shift to lower levels as the light intensity is reduced being a striking feature of the mode of interaction between light intensity and nitrate supply in the present series of experiments with *Lemna* colonies (White, 1937a). *This effect may well arise through a marked influence of light on reductase activity and the detrimental effect of excess nitrate on protein level.* It is noteworthy that *increase* of nitrate supply has been found to lead to *decrease* of protein level by Nightingale (1927) working with tomato and salvia plants subject to a short length of day.

These considerations favour the view that under low light intensity nitrate accumulation, presumably resulting from depression of growth rate in conjunction with relatively unchanged rate of nitrate supply and possibly retardation of reductase activity, leads to a virtual condition of nitrogen starvation

¹ Decrease in nitrate supply at 50 ft.-candles raises protein content to approximately the same level as at 300 ft.-candles (White, 1937a, Fig. 10).

through reduction in protein content. In association with this trend there is a tendency for the accumulation of surplus carbohydrate.¹

The interaction of potassium and light intensity.

The effect of increase of potassium supply from 2·0 to 200 mg. per litre at different light intensities may first be considered. At the maximal light intensity of 300 ft.-candles the rate of growth of the colony with higher potassium concentration (200 mg.), estimated from rate of increase in frond number (Table III), frond area (Table V), and depth of frond colour (Fig. 12), exceeds that of the colony with lower potassium concentration (2·0 mg.), whereas at the minimal experimental light intensity of 60 ft.-candles the reverse relation holds. The optimal potassium concentration for growth is thus shifting to higher levels as the light intensity is increased and to lower levels as the light intensity is decreased. These effects are conformable with the following considerations. Growth-rate is decreased by reduction of light intensity at all potassium levels, and falling light intensity with unchanged potassium supply should therefore be associated with a relatively higher concentration of potassium in the frond. If the potassium supply were relatively high, as with 200 mg. per litre, symptoms associated with potassium excess would be induced or accentuated by reduction of light intensity. On the other hand, growth-rate is increased by increase of light intensity at all potassium levels, and rising light intensity with unchanged potassium supply should therefore be associated with a relatively lower concentration of potassium in the frond. If the potassium supply were relatively low, as with 2·0 mg. per litre, deficiency symptoms would be induced or accentuated by increase of light intensity.

The effect of reduction of potassium supply below 2·0 mg. per litre at different light intensities is not the same for rate of increase in frond number as for assimilation rate. At all light intensities 2·0 mg. per litre remains a more favourable concentration for rate of increase in frond number than lower levels of potassium supply (Table II), whereas assimilation rate, on the other hand, is minimal with minimal potassium supply at high light intensity but maximal with minimal potassium supply at low light intensity (Table VII). In order to account for these effects attention is directed to the following considerations. In general the effect of decrease in light intensity is to reduce the severity of potassium deficiency so that the detrimental effect of lack of potassium tends to disappear at low light intensity (see pp. 622, 629, 632). Fall in rate of increase in frond number (Table II), increase in dry weight per unit area (Fig. 8), and decrease in amylolytic activity (Table VIII) indicate that the colony without potassium is showing symptoms of deficiency

¹ The accentuation of this carbohydrate accumulation and depression of growth rate in the present experiment as compared with that reported previously (White, 1937a) may be attributed to the higher nitrate concentration used in the present experiment (20 mg. nitrogen per litre as against a maximum of 4 mg. per litre).

of this element even at a light intensity as low as 60 ft.-candles. The corresponding increase of net assimilation rate, which arises as a result of a relative increase in frond dry weight of the colony without potassium (see p. 629) despite a falling rate of increase in frond number, signifies that the optimal potassium concentration for the process of assimilation is lower than for rate of increase in frond number. This is confirmed by the delay in fall of assimilation rate at higher light intensity (Fig. 14), immediately following transference of the colony to a nutrient solution lacking potassium. The effects of variation in potassium supply at different light intensities are thus conformable with the view that colonies, after the same period of low potassium supply, are in a condition corresponding with severe potassium starvation at high light intensity and slight potassium deficiency at low light intensity.

Turning to the effects of variation in light intensity at different levels of potassium supply, reference may be made to the values for frond area. The curves for frond area plotted against light intensity show stages of transition from a rising curve with relatively high potassium supply to well-defined 'optimum' curves with low potassium supply (Fig. 6). These results are conformable with the view that the influence of decrease in light intensity with constant potassium supply is twofold: (1) the detrimental effect of falling light intensity, (2) the favourable effect of rising potassium concentration in the frond resulting from decrease in growth-rate with unchanged potassium supply. The magnitude of this latter effect should clearly be accentuated by decrease of potassium supply and diminished by increase of potassium supply. If rate of growth of the frond in area were related to both effects maximal frond area would obtain at the point of balance between them, which would correspond with higher light intensities as the potassium supply was increased and lower light intensities as the potassium supply was decreased. By progressive increase of potassium supply a rising curve would obtain, for the 'maximum' of this curve would shift to correspond with a potassium concentration outside the range of those used in the experiment. By progressive decrease of potassium level the 'maximum' of the curve relating frond area to light intensity would shift in the opposite direction, so that if potassium level was reduced low enough maximal frond area would be associated with minimal light intensity. Confirmation of this trend may be seen in Fig. 5, which shows that with progressive decrease of potassium supply maximal frond area is obtained at progressively lower light intensity.

SUMMARY

1. A study has been made under controlled conditions of the effect on the growth and assimilation of *Lemna minor* of all combinations of four light intensities (300, 180, 120, and 60 ft.-candles continuous illumination) and four levels of potassium supply (200, 2·0, 0·125, and 0·0 mg. per litre).
2. Significant correlation coefficients for the sixteen combinations of light

intensity and potassium concentration are found between: dry weight per unit area and starch content ($r = +0.810$), frond area and protein level ($r = +0.896$), protein level and depth of frond colour ($r = +0.888$). The fluctuations in frond area that are associated with variation in potassium supply are inverse to the corresponding fluctuation in dry weight per unit area.

3. The metabolism of potassium-starved colonies is considered. The immediate result of transference of a colony to a potassium-free solution is a decrease in frond area (and in root length) and increase in dry weight per unit area. These effects precede fall of assimilation rate and are attributed to decrease in water-content. Subsequently potassium deficiency is associated with a decrease in assimilation rate, protein level, rate of increase in frond number, and amylolytic activity. There is a tendency for starch level to rise, presumably at the expense of sugars, in the potassium-starved frond and with this effect is associated the fall in water content. The optimal potassium concentration for assimilation rate, frond colour, and possibly protein content, which is invariably correlated with frond colour, is appreciably lower than for rate of increase in frond number or frond area.

4. Dry weight per unit area decreases with reduction in light intensity but reaches a minimal level at about 130 ft.-candles, further decrease in light intensity from 120 to 60 ft.-candles being associated with a rise in dry weight per unit area in spite of continued fall in assimilation rate. The results obtained are conformable with the view that at low light intensity growth is controlled by decreasing protein level rather than by the balancing of assimilates by respiration losses.

5. With the highest light intensity of 300 ft.-candles maximal rates of increase in frond number and area are obtained with the highest potassium concentration of 200 mg. per litre. With the lowest light intensity of 60 ft.-candles maximal rates of increase in frond number and area are obtained with 2.0 mg. potassium per litre. Frond dry weight and net assimilation rate (increment in dry weight per unit time) are minimal with lowest potassium supply at the highest light intensity and maximal with lowest potassium supply at the lowest light intensity. These effects are conformable with the following considerations. The detrimental effect of low potassium level is accentuated by increase of light intensity, which is associated with increased growth rate and presumably relatively lower potassium concentration in the frond. The detrimental effect of potassium excess is accentuated by decrease of light intensity, which is associated with decreased growth-rate and presumably relatively higher potassium concentration in the frond.

6. Rate of increase in frond number, frond dry weight and net assimilation rate fall with decrease of light intensity at all levels of potassium supply. Protein level (as estimated by Millon's colour test) and depth of frond colour fall with decrease of light intensity at high potassium level but rise with decrease of light intensity at low potassium level. With high potassium supply a rising curve relating frond area to light intensity is obtained, whereas

with low potassium supply 'optimum' curves are obtained. These results are conformable with the view that the effect of decrease in light intensity with constant potassium supply is twofold: (1) the detrimental effect of falling light intensity, (2) the favourable effect of rising potassium concentration in the frond, resulting from decrease in growth-rate. The intensity of this latter effect is accentuated by decrease and diminished by increase of potassium supply. Maximal frond area obtains at the point of balance between these effects, which corresponds with higher light intensities as the potassium supply is increased and with lower light intensities as the potassium supply is decreased.

Grateful acknowledgement is due to Professor F. G. Gregory, with whom the author has been privileged to discuss many points arising in the presentation of the results.

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Studies in the Nutrition of Vegetables

The Effects of Variation in the Nitrogen Supply on Lettuce (var. May King) in Sand Culture

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EXPERIMENTAL

THE glazed culture-jars used each held about 46 lb. of a fine, white, pure, silica sand previously described (Woodman, 1936). To prevent escape of this sand, the inner ends of the drainage-tubes were arranged to be flush with the inner ends of the waxed corks through which they ran, and glass-wool was then secured over these ends by trapping it between the corks and the tubular apertures of the jars when the corks were inserted.

Two experiments were made. In each the nitrogen applied was varied. The concentrations of the important elements present in the media used are given in Table I. Iron was always present as ferrous sulphate heptahydrate (Analar). The salts used in making the media were: sodium nitrate (Analar) as the source of nitrogen; anhydrous disodium phosphate (Analar) as the source of phosphorus; potassium sulphate (Analar) as the source of potassium and sulphur; calcium chloride (solution), made by neutralization of calcium carbonate (Analar) with hydrochloric acid, as the source of calcium; and magnesium sulphate heptahydrate (Analar) as the source of magnesium and sulphur. The minor elements were found to be unnecessary with these cultures.

In the first experiment 7 media, A-G, were used; there were 12 replications of the first 5 treatments, A-E, and 3 of F and G, making a total of 66 cultures. Treatments A-E were arranged in a greenhouse maintained at 50° F. as a 'layout' of 12 randomized blocks, and the 6 cultures with F and G were placed at the end of this randomized-block arrangement.

TABLE I

Medium	Concentration (p.p.m.) of certain elements in the medium						
1st experiment:	N	P	K	Ca	Mg	Fe	S
A	32.96	21.85	22.44	9.03	5.05	0.50	16.15
B	16.48	"	"	"	"	"	"
C	6.18	"	"	"	"	"	"
D	2.06	"	"	"	"	"	"
E	0.41	"	"	"	"	"	"
F	nil	"	"	"	"	"	"
G	16.48	nil	nil	nil	nil	,,	0.29

2nd experiment:	N	P	K	Ca	Mg	Fe	S
A	32.96	21.85	44.88	9.03	5.05	0.50	25.35
B	16.48	"	"	"	"	"	"
C	6.18	"	"	"	"	"	"
D	2.06	"	"	"	"	"	"
E	0.41	"	"	"	"	"	"
F	nil	"	"	"	"	"	"

Sowing of the lettuce (May King) (the sample of seed used has been previously described) was on September 7, 1936, in the sand in the culture jars, so that the seed actually germinated in the presence of the desired medium, and there was no transplanting to check growth and bring on the coloration of this tinted lettuce (1936). Germination took place from September 11 to 13, and the seedlings were thinned out on the 15th to leave five equally spaced seedlings for five harvests (1936). During the whole course of the experiment 14,450 c.c. of medium were given to each culture, usually in 300, 400, or 500-c.c. lots, four times a week.

In the second experiment the 'nitrogen-alone' medium G was omitted. Six media, A–F, were therefore used; and as F (no nitrogen) was now made a full treatment, there were $6 \times 12 = 72$ cultures. The 'layout' was again a randomized-block arrangement with 6 treatments and 12 replications. The sowing (the same sample of seed again) was made on December 12, 1936. Germination took place from December 19 to 21, and the seedlings were singled to leave one central seedling only on the 24th, as no seedling harvests were to be taken. The total volume of medium applied to each culture during the whole of the experiment was 16,700 c.c., mostly in 300, 400, or 500-c.c. lots, four times weekly. The media in this second experiment contained twice the amount of potassium in the first.

OBSERVATIONS ON THE CULTURES

Notes on the first experiment.

September 7, 1936: day of sowing of lettuce. September 11–13, 1936: germination. September 15, 1936: the seedlings were thinned to 5 per pot. September 16, 1936: the cultures dripped freely on addition of fresh medium. The dates of the four seedling harvests and the main one were September 24 and 30, October 5 and 12, and November 27, 1936, respectively.

Notes made during the course of the experiment are summarized below:

Tinting of cultures. An adequate supply of nitrogen in the seedling stages gave green plants, less nitrogen caused anthocyan tints, and a deficiency or total absence gave green seedlings. As the plants matured, anthocyan pigment developed with deficiency or total absence of nitrogen, so that these cultures (E and F) were ultimately the most tinted plants. Cultures which received nitrogen only were always purple.

Scorch. The severity of scorch appeared finally to follow the order A > B > C > D < E. Green plants seemed to be more susceptible to

scorch than tinted ones, and the A and B plants were very severely scorched. Cultures with E were worse than the less-tinted D cultures, however, but this was possibly due to the fact that they developed scorch during the preliminary green period mentioned above.

It was thought that an insufficient supply of potassium might have caused this severe scorch, and this was one reason for the second experiment, with twice the quantity of potassium in the media.

Size. Weekly linear measurements of the cultures were made: the greatest breadth, and the breadth at right angles to this. In the early periods of growth the order of size was A > B > C > D < E < F > G. The results for both F and G were the means of three plants only and possibly need no explanation, therefore. The anomalous position of E was probably due to the fact that the seedlings were green at this stage, and green plants tended to be larger than tinted ones even within the same treatment; later the E plants assumed the expected order, so that D became > E. Nitrogen alone (treatment G) gave very stunted cultures.

Hearting. At the main harvest there were 5 of A fully hearted and the other 7 showed distinct signs of hearting; 4 of B showed distinct signs; none of the others showed signs. An adequate supply of nitrogen, therefore, tended to earlier maturity.

Roots. The roots at the main harvest were normal tap roots with the usual accompanying fibre, and were progressively smaller with decrease in the supply of nitrogen, except for the no-nitrogen ones, which were fairly large (there were three cultures only of these, however).

Grade. Owing to the presence of scorch none of the lettuces could be classed as Grade I.

Notes on the second experiment.

In this experiment the amount of potassium was double that of the first to see if scorch could be prevented. As the 3 cultures with the no-nitrogen treatment in the first experiment gave large plants for some reason, this treatment (F) was here expanded to a full treatment of 12 cultures. The G (nitrogen-alone) cultures were omitted. There were thus 72 cultures. No seedling harvests were made.

December 12, 1936: sowing in culture jars. December 19-21, 1936: germination. December 21, 1936: the cultures were dripping freely with the medium. December 24, 1936: the cultures were singled so that one (central) seedling was left in each jar. April 8, 1937: harvest.

The following is a summary of notes made during the course of the experiment.

Tinting of cultures. Throughout the whole course of the experiment a progressive decrease in nitrogen resulted in a progressive increase in anthocyan tinting; the intensity of tinting thus increased continuously through A, B, C, D, E, and F (no nitrogen), A and B yielding green cultures.

In general also, a deficiency of nitrogen induced bleaching of the older leaves and of the green portions of the plants. The older leaves were eventually bleached to a golden yellow, and many died prematurely; the green portions of the plants had a 'bleached-green' look, the green thus appearing lighter in shade than for the A and B cultures.

Scorch. There was practically no scorch. This might have been due to the extra potassium, or to the season of the year (winter and early spring).

Size. The size of the cultures as measured by the usual linear measurements went down regularly with decrease in the nitrogen supply from A to F.

Hearting. On the day of harvest the 12 cultures with treatment A showed distinct signs of hearting, and 2 with B; none of the others showed signs. Good supplies of nitrogen thus tended to earlier maturity, as was demonstrated also by the first experiment.

Roots. The roots on the day of harvest were all normal with the usual fibre. Decrease in nitrogen caused a smaller root with less fibre.

Grade. All 24 cultures with the A and B treatments were Grade I, though not fully hearted. Other lettuces were tinted and immature.

THE YIELD WITH DIFFERENT TREATMENTS

Values obtained for each culture were the fresh weights of the top (cut off at the junction of top with stalk), root, and whole plant, the corresponding dry weights, the top/root ratio for fresh and dry weights, and the percentage moisture contents of the fresh material of the top, root, and whole plant.

The actual data of the experiment were too bulky to be reproduced, but are available for examination on request in this laboratory. The analyses of variance were worked out for the fresh and dry weights of the tops, roots, and whole plants, the top/root ratios for fresh and dry weights, and the percentages of moisture in the tops, roots, and whole plants. The corresponding summaries of results are tabulated in Tables II and III, together with the standard errors of the treatment means (Wishart and Sanders, 1935). Arithmetical and not weighted means have been used when dealing with ratios and percentages.

TABLE II
Summaries of Results

Description of data. 1st experiment. F.W.}	Treatment mean.	Treatment:					Mean.	S.E.	No nitro- gen. alone.	
		A.	B.	C.	D.	E.				
Tops }	I. Yield (gm.)	0·202	0·160	0·107	0·043	0·026	0·1076	0·00830	0·024	0·028
F.W. }	SSS.	A>B>C>D = E.								
	II.	0·659	0·532	0·232	0·087	0·066	0·3152	0·03015	0·112	0·047
	SSS.	A>B>C>D = E.								
	III.	1·506	1·017	0·366	0·186	0·097	0·6344	0·05224	0·182	0·037
	SSS.	A>B>C>D = E.								
	IV.	3·535	2·240	0·690	0·293	0·191	1·390	0·1168	0·580	0·058
	SSS.	A>B>C>D = E.								
	V.	37·68	26·67	6·56	2·80	2·63	15·27	1·436	5·12	0·070
	SSS.	A>B>C = D = E.								

Variation in the Nitrogen Supply on Lettuce (var. May King) 653

Description of data. 1st experiment.	Treatment mean.	Treatment:					Mean.	S.E.	No nitro- gen.	Nitro- gen alone.
		A.	B.	C.	D.	E.				
Tops, moisture	I.	93·73	94·53	91·77	91·64	93·78	93·09	0·2887	81·16	80·53
	SSS.	A = B = E > C = D.								
	II.	93·54	92·14	90·80	90·42	89·24	91·23	0·2852	93·52	90·87
	SSS.	A > B > C = D > E.								
	III.	91·74	90·93	89·71	88·85	89·95	90·24	0·2954	89·76	85·96
Roots, moisture	SSS.	A = B > C = E; E > D; C = D.								
	IV.	92·51	91·59	89·72	86·72	85·98	89·30	0·7069	91·25	80·33
	SSS.	A = B > D = E; A > C; B = C > D = E.								
	V.	93·45	93·17	91·59	89·12	86·38	90·74	0·6668	88·94	79·31
	SSS.	A = B = C > D > E.								
Whole plants, moisture	I.	80·18	78·32	75·13	76·89	84·43	78·99	0·9683	73·61	53·70
	SSS.	E > A = B > C; A = D; B = D.								
	II.	88·95	84·47	80·29	82·01	68·35	80·81	1·046	85·99	84·20
	SSS.	A > B > C > E; B = D; C = D; A > D > E.								
	III.	83·19	77·96	78·24	80·71	88·06	81·63	0·7803	86·12	82·65
Whole plants, moisture	SSS.	E > A > D > B = C.								
	IV.	92·95	90·22	86·64	83·51	80·25	86·71	0·6966	81·43	68·51
	SSS.	A > B > C > D > E.								
	V.	88·67	89·27	85·99	80·67	88·45	86·61	1·237	87·41	86·22
	SSS.	A = B = C = D > E.								

TABLE III
Summaries of Results

Description of data. 2nd experiment.	Treatment mean.	Treatment:					Mean.	S.E.		
		A.	B.	C.	D.	E.				
Tops	F.W.	Yield (gm.)	54·0	30·9	10·29	3·29	1·73	16·89	1·387	
		SSS.	A > B > C > D = E = F.							
Roots	F.W.		10·24	11·55	7·13	2·59	1·59	5·605	0·5478	
		SSS.	A = B > C > D = E; D > F; E = F.							
Whole plants	F.W.		64·23	42·41	17·42	5·88	3·32	22·48	1·796	
		SSS.	A > B > C > D = E = F.							
Tops	D.W.		4·30	2·88	1·28	0·431	0·281	1·559	0·1237	
		SSS.	A > B > C > D = E = F.							
Roots	D.W.		1·36	1·72	0·74	0·302	0·179	0·7288	0·1014	
		SSS.	B > A > C > D = E = F.							
Whole plants	D.W.		5·65	4·60	2·02	0·733	0·460	2·286	0·2003	
		SSS.	A > B > C > D = E = F.							
Top/root	F.W.	Ratio	5·35	2·76	1·55	1·33	1·10	2·53	2·437	0·2011
		SSS.	A > B = F > C = D = E.							
Top/root	D.W.		3·38	1·86	1·87	1·59	1·91	1·82	2·072	0·2588
		SSS.	A > B = C = D = E = F.							
Tops, moisture (%)		%	91·92	90·70	87·56	86·98	82·61	81·94	86·95	1·232
		SSS.	A > C = D > E = F; A = B > D > E = F; B = C; C = D > E = F.							
Roots, moisture (%)			86·95	85·44	89·62	88·63	89·28	86·82	87·79	0·8492
		SSS.	C = D = E; C > A = B = F; E = A = F; E > B; D = A = F; D > B.							
Whole plants, moisture (%)			91·14	89·26	88·37	87·69	85·70	83·82	87·66	0·8422
		SSS.	A = B > E = F; A > C = D; B = C = D; C > E = F; D = E; D > F.							

Key to the Tables of Summaries of Results

The first column is 'Description of Data'. 'F.W.' and 'D.W.' denote fresh and dry weights; the figures I, II, III, IV, and V, the first, second, third, and fourth seedling-harvests, and the main harvest, for the first experiment.

In the first column there occurs, for each summary of results, the description SSS, which indicates that the results (treatment means for that sub-table) are highly significant according to Fisher's Tables of z at the 0·1% level. The value of z obtained from the analyses of variance was $\frac{1}{2} \log_e$ (treatment mean square) minus $\frac{1}{2} \log_e$ (error mean square).

The second column describes the 'Treatment mean', whether it is a yield, a ratio, or a percentage; the actual treatment mean is recorded under the 'Treatment' column sub-headings A, B, C, &c. The mean of the whole of the results for a sub-table (which is also the mean of the treatment means) is given in the 'Mean' column. The Standard Error of the treatment means is recorded in the column labelled 'S.E.'

In the second line of each sub-table is given a summary of the comparisons of significance carried out on the treatment means. The signs '=' and '>' are to be read as 'not significantly different from' and 'significantly greater than', respectively.

The 'No-nitrogen' and 'Nitrogen-alone' results for the first experiment are averages of three cultures for each treatment and are tabulated in the two columns after the 'S.E.' column. Only the results for treatments A-E were included in the randomized-block system, and the Mean and S.E. apply to these five treatments. In the second experiment the 'No-nitrogen' treatment was expanded to a full one, F, taking part in the randomized arrangement, so that in Table III the six treatments A-F precede the 'Mean' column.

DISCUSSION OF RESULTS

As the concentration of potassium in one set of media was twice that in the other, with a corresponding difference in the ratios of potassium to the other elements, and as the seasons of growth for the two sets of cultures were different, exactly parallel results could not be expected. Nevertheless, there was a great similarity between the results of the two experiments for the mature lettuce, particularly if too much attention were not paid to treatments which gave small yields and which were, therefore, commercially unimportant.

The results of the experiment were highly significant. The following conclusions are for the mature crop in the main.

Fresh weights of plants. The results of the two experiments indicate that increase in the supply of nitrogen as nitrate caused increased yield of *tops*, besides the earlier maturity previously mentioned. In both experiments treatments A and B were far superior to C and D, and A was the best and would be aimed at commercially. Somewhat similar results were obtained for the fresh weights of the *roots* and *whole plants*, though it was evident from the results for treatments A and B that nitrogen was more necessary for tops than for roots.

Dry weights of plants. Similar results were obtained as for the fresh weights, and the tendency for the tops rather than the roots to profit from heavy doses of nitrogen was again evident; thus in the second experiment treatment A, with the greatest amount of nitrogen, significantly depressed the yield of dry matter for the roots, and, in the first, this treatment was only equal to B.

Top/root ratios. These ratios for the *fresh weights* of the mature lettuce were, in general, lowered by a decrease in the nitrogen applied; that is, nitrogen increasingly favoured the top rather than the root, the more that was applied. A somewhat similar conclusion could be drawn from these

ratios for the *dry weights*, treatment A giving the greatest ratio for both sets of mature lettuces.

Moisture in the plants. In both experiments for the *tops* there was in general a progressive decrease in percentage moisture content with decrease in the nitrogen applied. The differences were not always significant, however; thus in experiment one the first three treatments and, in the second, the first two, gave statistically-equal moisture contents. The variations between the treatments resulted also in small differences only in the moisture contents of the *roots* and the *whole plants*, the values for the whole plants decreasing progressively in both experiments for the mature lettuce in a very similar manner to those for the tops.

SUMMARY

A progressive reduction in the amount of nitrogen applied as sodium nitrate to sand-cultures of lettuce (*var. May King*) resulted in a progressive reduction in size and weight, a progressive development of anthocyan pigment, and finally a tendency to etiolation of the remaining green portions of the leaves with sometimes formation of a golden-yellow colour in the oldest leaves.

An adequate supply of nitrogen tended to give earliness of maturity as shown by 'hearting', and to favour the growth of tops rather than of roots.

I thank my assistants, Messrs. T. W. McKean and J. F. Leonard, for their great help during the course of this experiment.

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The Nature of the Accessory Growth Factors influencing Growth and Fruiting of *Melanospora destruens* Shear. and of some other Fungi

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I. INTRODUCTION

IT is now well known that many fungi and bacteria are unable to grow satisfactorily in synthetic media containing sugar and salts unless small quantities of certain other substances, known as accessory growth factors or growth substances, are added. This was first pointed out by Wildiers (1901), who showed that a particular yeast which did not grow in a synthetic medium when the amount of inoculum was small, grew well if a larger inoculum were used. A small inoculum, however, made good growth in a similar medium if a quantity of a boiled suspension of yeast were added. Wildiers concluded that a chemical substance present in the yeast suspension was necessary for good growth. He called this substance 'bios'.

Wildiers' work was criticized at the time, and it was not until comparatively recently that its importance was recognized. Early work on the subject has been summarized by Tanner (1925).

The nature and activity of 'bios' has been extensively studied, and it was soon realized that in some cases the so-called 'bios' effect was not due to a

single substance but to a complex of growth substances. Lucas (1924) succeeded in fractionating the 'bios' contained in malt combings and rice polishings into two parts which he called Bios I and Bios II, both of which were essential for good growth of a strain of *Saccharomyces cerevisiae*. Later Bios I was identified with inositol by Eastcott (1928), and a further fractionation of Bios II into at least two essential parts was described by Miller et al. (1932).

Buston and Pramanik (1931) showed that the accessory factor found by Farries and Bell (1930) to be necessary for growth of *Nematospora gossypii* could be fractionated into at least two parts, one of which they identified with inositol. Miller (1934) later showed that inositol, Bios II_A, and Bios II_B when added to Buston and Pramanik's medium gave good growth of the related species *Nematospora coryli*.

Meanwhile a large amount of data has been accumulated by various workers, indicating that vitamin B₁ (aneurin of European writers, thiamin of later American writers) has growth-promoting activity with certain fungi and bacteria.

Williams (1919), working with a commercial strain of yeast which may not have been in pure culture, suggested that the increased growth in the presence of milk, wheat germ, &c., was due to their vitamin B content. Later (Williams and Roehm, 1930) he concluded that vitamin B₁ was one of the 'bios' substances necessary for his strain of yeast. Lepeschkin (1924) also found that vitamin B was essential for the growth of some yeasts and showed that it also increased the growth of *Penicillium glaucum*.

Schopfer (1931, 1932) described a striking increase in the number of zygosporae formed by *Phycomyces Blakesleeanus* in the presence of a vitamin B preparation or of an extract of germinating wheat. The stimulatory effect of vitamin B on growth of *P. Blakesleeanus* was also noted by Burgeff (1934). Later Schopfer (1934a, 1934b, 1935a) showed that the addition of crystalline vitamin B₁ to a synthetic medium stimulated growth of this fungus and of some other members of the Mucorineae (1935b). A mixture of the thiazole and pyrimidine components of aneurin could replace the vitamin itself for growth of *Phycomyces* but closely allied compounds were inactive (Schopfer, 1937). Schopfer extended this study to a number of other organisms and grouped them according to whether they required aneurin as such or one or both of its components (Schopfer, 1938a, b).

Robbins and Kavanagh (1937a, b) and Sinclair (1937) confirmed Schopfer's statement that *P. Blakesleeanus* required vitamin B₁ or both intermediates. Robbins (1937) has also described some fungi which would grow well in the absence of thiazole but needed an external supply of pyrimidine. The specificity of the thiazole and pyrimidine intermediates of aneurin for growth of *Phycomyces* has been demonstrated (Robbins and Kavanagh, 1938a, b).

The importance of vitamin B₁ in the metabolism of certain wood-destroying fungi has been indicated by Noecker (1938).

Vitamin B₁ and in some instances its thiazole and pyrimidine components

have been shown to stimulate growth of some bacteria (Tatum et al., 1936; Knight, 1937a, b; and Knight and McIlwaine, 1938).

The study of the bios group of growth substances was taken up by Kögl and his co-workers and their work has been summarized by Kögl (1938). Kögl and Tönnis (1936) isolated from egg-yolk a crystalline substance which they termed 'biotin' which greatly increased the growth of a strain of yeast. Growth was further increased by the addition of inositol which was isolated from the Bios I fraction of yeast by Kögl and van Hassett (1936) and of aneurin (vitamin B₁) (Kögl, loc. cit.). Later Kögl and Fries (1937) showed that inositol and biotin were essential for good growth of *N. gossypii* on Busto and Pramanik's medium and that the addition of aneurin further increased the growth. Certain other fungi, e.g. *Phytophthora cactorum*, *Polyporus adustus*, &c., needed only aneurin. Fries (1938) states that while certain wood-destroying fungi and some others require only aneurin, both aneurin and biotin are required for maximum growth by *Lophostomium pinastri*, *Hypoxyylon pruinatum*, and *Melanconium betulinum*.

In two previous papers (Asthana and Hawker, 1936, and Hawker, 1936) the writer has described the stimulatory effect of media stale by certain fungi and of crude lentil extract (as prepared by Busto and Pramanik, loc. cit.) on the growth and fruiting of *Melanospora destruens*. In view of the recent advances in the study and identification of fungal growth substances described above the effects on Melanospora of certain pure preparations were investigated. A preliminary account of the more striking results of this investigation has already been published (Hawker, 1938).

II. EXPERIMENTAL METHODS

The methods used were in general those of the previous papers, with the exception that the small quantity of some of the pure substances available enforced severe economy in the amount of medium used. Experiments were run in triplicate on small-sized Petri dishes (6 or 9 cm. diam.). The number of repeat experiments was also necessarily reduced, but each experiment was repeated at least once, sometimes in a slightly modified form.

The pure substances tested were as follows:

- (1) *Biotin*, 25 γ (= 0.025 mg.) of pure biotin methyl-ester and a larger quantity of an impure preparation obtained from Professor F. Kögl of Utrecht.¹
- (2) *Aneurin* (vitamin B₁), a pure crystalline preparation from Hoffmann-La Roche & Co.
- (3) *Thiazole and pyrimidine components of aneurin*, pure preparations of 4-methyl-5-β-hydroxyethyl thiazole and 2-methyl-4-amino-5-amino-methyl pyrimidine hydrochloride, obtained from Dr. B. C. J. G. Knight.¹
- (4) *Inositol*, a pure preparation from British Drug Houses, Ltd.

¹ The writer is deeply indebted to Professor F. Kögl and Dr. B. C. J. G. Knight for kindly supplying these preparations.

These were added in various quantities to the synthetic medium (medium A—glucose, 5 gm.; KNO_3 , 3.5 gm.; MgSO_4 , 0.75 gm.; KH_2PO_4 , 1.75 gm.; agar, 15 g.; water, 1 litre) used in previous work or to other media as stated in the text. Sterilization was carried out at 100°C . on three successive days, and the cultures were incubated at 25°C . in the case of *M. destruens* and at 20°C . or 25°C . with certain other fungi.

Perithecial frequency of Melanospora was measured on agar plates by the method previously described (Asthana and Hawker, loc. cit.). Dry weights of mycelia were obtained from parallel liquid cultures in medicine bottles each containing 20 c.c. medium which formed a thin layer when the bottles were laid on their sides (Hawker, 1939).

III. EFFECT OF BIOTIN, ANEURIN AND ITS COMPONENTS, AND INOSITOL ON *M. DESTRUENS*

The following agar media were prepared:

- (1) Medium A
- (2) " + inositol (20 mg. per 100 c.c. medium)
- (3) " + biotin (4 γ " ")
- (4) " + aneurin (4 γ " ")
- (5) " + biotin + inositol
- (6) " + aneurin + inositol
- (7) " + biotin + aneurin
- (8) " + biotin + aneurin + inositol
- (9) " + biotin + thiazole (equivalent to that present in 4 γ aneurin per 100 c.c.)
- (10) " + biotin + pyrimidine (equivalent to that present in 4 γ aneurin per 100 c.c.)
- (11) " + biotin + thiazole + pyrimidine
- (12) " + inositol-free fraction of lentil extract equivalent to 0.1 per cent. (by dry weight) crude extract.

The quantity of biotin available did not permit the preparation of a complete parallel series of liquid media, but these were prepared for media 1-4, 7, 8, and 12. Media 3, 5, and 7 were also made up with pure biotin methyl-ester and these gave similar results.

Table I gives the results of this experiment. The strain of Melanospora used gave negligible growth in liquid medium A and only a very thin colony of diameter 1-3 cm., with an irregular margin, on agar medium A. The slight growth on the latter was probably due to minute quantities of growth substances in the agar. Addition of inositol or aneurin (Pl. XXI, Fig. 1) had no effect. Melanospora grew well in both liquid and solid media when biotin was added (Pl. XXI, Fig. 2). On agar plates the hyphae were freely branched and reached the edge of the plate, but no perithecia were formed on agar or in liquid medium even when cultures were kept for two to three months.

TABLE I

Medium.	Dry wt. mycelium (mg. per 100 c.c. medium after 1 week) (liquid medium).	Perithecial frequency after 1 week (agar medium).
(1) A	negligible	none
(2) ,, + inositol	”	”
(3) ,, + biotin	24	”
(4) ,, + aneurin	negligible	”
(5) ,, + biotin + inositol	—	”
(6) ,, + aneurin + inositol	—	”
(7) ,, + biotin + aneurin	165	4·6
(8) ,, + biotin + aneurin + inositol	162	4·8
(9) ,, + biotin + thiazole	—	none
(10) ,, + biotin + pyrimidine	—	4·3
(11) ,, + biotin + thiazole + pyrimidine	—	4·7
(12) ,, + inositol-free fraction of lentil extract	142	1·9

When both biotin and aneurin were present growth was very much increased and numerous perithecia were formed (Pl. XXI, Fig. 3). Growth and fruiting were superior to that in the presence of 0·1 per cent. of the inositol-free fraction of lentil extract.

The addition of inositol to media containing biotin or aneurin or both these growth factors had no significant effect on growth or fruiting. It has already been recorded (Hawker, 1936) that the addition of inositol to the inositol-free fraction of lentil extract did not increase the spore-promoting activity of the latter.

In the presence of biotin, aneurin could be replaced by a mixture of the thiazole and pyrimidine components or by pyrimidine alone, but not by thiazole alone. In the absence of biotin these substances were inactive.

The failure of the fungus to grow and fruit on medium A with the addition of aneurin was an unexpected result, since in an earlier experiment (Hawker, 1936) the addition of aneurin stimulated perithecial formation although it was not so effective as lentil extract in this respect. Table I (7 and 8) shows that the sample of aneurin used in the present investigation is effective in the presence of biotin, hence the difference between the two results cannot be explained on the basis of the presence of inhibitory substances in the second sample of aneurin or to differences in the method of sterilization. It is possible that the first sample of aneurin contained traces of biotin as an impurity, but this is unlikely since the sample was also tested on *Nematospora* with negative results. Kögl and Fries (loc. cit.) have shown that this organism responds to minute quantities of biotin, and it is therefore unlikely that sufficient biotin to influence *Melanospora* was present. There remains the possibility that some change has taken place in the fungus since the date of the first experiment. It has been previously pointed out (Hawker, 1936, 1939) that during this series of investigations the strain of *Melanospora* used has shown a progressive loss of fertility. It is therefore possible that the fungus

was formerly able to synthesize biotin to a certain extent, but that this ability has since been lost. Addition of aneurin to medium A would be effective if the fungus were able to produce traces of biotin, but would be effective only in the presence of an external supply of the latter if the fungus had lost the power of synthesis. Such an hypothesis does not admit of direct proof, but it is of interest in this connexion to note that some saltant strains do respond to aneurin in the absence of an external supply of biotin. The amount of response varies with the strain.

The effects on growth and fruiting of *Melanospora* arising from different concentrations of biotin and aneurin were studied and the results of two such experiments are given in Tables II and III. In the experiment recorded in Table III pure biotin methyl-ester, in the other the less pure preparation, was used.

TABLE II

Biotin (γ per 100 c.c. medium).	Aneurin (γ per 100 c.c. medium).	Perithecial frequency After 8 days.	Perithecial frequency After 13 days.
4·0	0	0	0
"	0·1	2·5	3·0
"	1·0	3·2	3·2
"	4·0	~	3·3
"	10·0	3·3	3·3
0	4·0	no growth	no growth
0·1	"	few	1·1
1·0	"	1·5	1·7
4·0	"	3·3	3·3
10·0	"	3·2	3·3

TABLE III

Biotin (γ per 100 c.c. medium).	Aneurin (γ per 100 c.c. medium).	Perithecial frequency After 7 days.	Perithecial frequency After 18 days.	Mycelial dry wt. (mg. per 100 c.c. medium).
0·0	0·0	no growth	—	—
0·0	4·0	"	—	—
0·1	0·1	few	0·4	70
0·1	4·0	"	1·4	132
4·0	0·0	0·0	0·0	17
4·0	0·1	0·0	1·0	95
4·0	4·0	1·7	2·6	191

These results show that the dry weight of mycelium and the number of perithecia formed are both influenced to some extent by the amount of either biotin or aneurin present. The most striking fact is that these growth substances are effective in very low concentration; as little as 0·1 γ per 100 c.c. medium (i.e. 1 part in 10⁹) of either substance gives a marked stimulation in the presence of an adequate supply of the other. When both are present in low concentration perithecial formation and probably mycelial dry weight are less than when the concentration of either is low and that of the other is relatively high (cf. lines 3, 4, and 6 of Table III). It is possible that the

fungus synthesizes small quantities of either growth substance in the presence of an adequate supply of the other. Further evidence supporting this suggestion will be given below. Table II shows further that a rise in concentration of biotin above 4 γ per 100 c.c. in the presence of 4 γ aneurin per 100 c.c. or of aneurin above 1 γ per 100 c.c. in the presence of 4 γ biotin per 100 c.c. does not produce any further increase in fruiting. This may be due to the limiting effect of concentration of the complementary growth substance or of food substances such as glucose. Owing to shortage of biotin these points could not be further investigated.

IV. INFLUENCE OF BIOTIN AND ANEURIN ON THE AMOUNT OF GLUCOSE OPTIMAL FOR SPORULATION OF *M. DESTRUENS*

In a previous paper (Hawker, 1936) it was demonstrated that the amount of glucose optimal for sporulation of *M. destruens* and of some other fungi could be raised by increasing the concentration of growth substances supplied. A few experiments were therefore carried out to determine whether biotin or aneurin or both were responsible for this effect.

Medium A was prepared with the normal amount of glucose (0.5 per cent.) and with 1.0 per cent. and 2.0 per cent. respectively. To quantities of each of these media growth substances were added as shown in Table IV. The results show that at either level of biotin an increase in amount of aneurin increases the number of perithecia formed, this being most marked at the higher concentrations of glucose. While an increase in biotin concentration at either level of aneurin produces a marked response on medium A (0.5 per cent. glucose), this is not seen when the percentage glucose is increased. Thus the amounts of glucose optimal or limiting for perithecial production are raised by increased aneurin concentration but are little affected by that of biotin.

TABLE IV

Biotin (γ per 100 c.c. medium).	Aneurin (γ per 100 c.c. medium).	Glucose		
		0.5 %	1.0 %	2.0 %
1	1	1.6*	0.9	0.0
1	4	1.9	2.3	0.9
4	1	2.4	0.4	0.0
4	4	2.6	2.8	0.6

* Figures refer to perithecial frequency 13 days after inoculation.

The effect of aneurin was studied in more detail in experiments in which biotin was added in the form of a small dose (0.05 per cent. by dry wt.) of lentil extract or medium in which *Rhizopus stolonifer* had grown and which was diluted to one-half. The concentration of glucose ranged from 0 to 2 per cent. and the amount of pure aneurin added from 0 to 10 γ per 100 c.c. A small quantity of aneurin which could not be estimated was necessarily added with the source of biotin so that the figures given in Table V are slightly

less than the actual amount of aneurin present. The results given in Table V, which refer to an experiment in which the source of biotin was medium on which Rhizopus had been grown, show that increase in concentration of aneurin tends to increase the amount of glucose optimal for sporulation.

TABLE V

Aneurin (γ per 100 c.c. medium).	Glucose				
	0 %	0.2 %	0.5 %	1.0 %	2.0 %
0	0.6*	2.8	2.6	few	0
1	0.7	2.9	3.0	2.4	few
4	0.8	2.8	3.9	2.5	1.2
10	0.7	2.6	4.0	4.8	3.1

* Figures refer to perithecial frequency 10 days after inoculation. Basal medium = a glucose asparagin medium used by Rhizopus plus an equal volume of water plus ingredients of medium A with the exception of glucose.

V. EFFECT OF BIOTIN, ANEURIN, AND INOSITOL ON SOME OTHER FUNGI

1. *Phycomyces nitens*. Growth on Kögl and Fries's medium (glucose, 20 g.; ammonium tartrate, 5 g.; ammonium nitrate, 1 g.; KH_2PO_4 , 0.5 g.; NaCl , 0.1 g.; FeCl_3 , 10 drops of a 1 per cent. solution; water, 1 litre) was increased from 25 mg. to 402 mg. dry weight per three flasks by the addition of 10 γ aneurin per 100 c.c. medium. Addition of the thiazole and pyrimidine components of aneurin to the same medium gave a similar increase, but either alone was ineffective.

These results confirm the findings of Robbins and Kavanagh (1938a, b) for this fungus and are similar to those of Burgeff (1934), Schopfer (1934a, 1937), Kögl and Fries (loc. cit.), Robbins and Kavanagh (1938b), and Sinclair (1937) for *P. Blakesleeanus*.

2. *Phytophthora cactorum*. Vegetative growth and production of oospores were increased by the addition of aneurin to medium A or to Kögl and Fries's medium, but a mixture of the thiazole and pyrimidine constituents was inactive. Biotin was also ineffective.

Kögl and Fries (loc. cit.) found that this fungus gave negligible growth in the absence of aneurin and was independent of an external supply of biotin. Robbins and Kavanagh (1937b) similarly found aneurin to be essential. Leonian (1936a, b), however, states that while this fungus reacts to certain natural products, the active factor could not be aneurin since the activity was retained after a treatment such as would destroy the vitamin. Robbins and Kavanagh (loc. cit.) suggest that *P. cactorum* may use the thiazole and pyrimidine components and that the aneurin molecule as such is not required. They showed that certain other species of *Phytophthora* were able to use these components. It was pointed out above that different strains of *Melanospora* reacted somewhat differently to certain growth substances, so that there is no necessary contradiction between Leonian's results and those of the writer.

3. *P. parasitica*. This species behaved in exactly the same manner as *P. cactorum* (see Pl. XXI, Figs. 4 and 5). Robbins and Kavanagh (loc. cit.) had previously shown that aneurin was essential for good growth of this fungus.

4. *Nematospora gossypii*. Inositol and biotin were found to be necessary for growth and a further increase was produced by the addition of aneurin.

The necessity of an accessory growth factor for good development of this fungus was first demonstrated by Farries and Bell (loc. cit.). Buston and Pramanik (loc. cit.) fractionated this growth factor into inositol and an inositol-free fraction. Recently Kögl and Fries (loc. cit.) demonstrated the necessity of inositol and biotin and the favourable effect of aneurin, with which the above is in complete agreement.

5. *Podospora curvula*. This fungus resembled *M. destruens* in that it produced only a scanty mycelium on medium A or medium A plus aneurin, grew well but did not fruit on medium A plus biotin, and grew well and produced numerous perithecia on medium A plus biotin plus aneurin.

6. *Sordaria* sp., *S. fimicola*. These behaved similarly to *Melanospora* and *Podospora*.

VI. EXPERIMENTS WITH MIXED CULTURES

Inhibitory or stimulatory effects of one organism upon another have been noted by a number of investigators.

The literature relating to the stimulation of sporulation of one fungus by another has been summarized in a previous paper (Asthana and Hawker, loc. cit.). The stimulatory effect on the fruiting of *M. destruens* of a number of fungi or the media in which they had grown was also described. Later (Hawker, 1936) it was shown that a synthetic medium in which *Botrytis cinerea* had been grown was thereby made suitable for the growth of *N. gossypii*, and was further improved by the addition of inositol.

Recently Kögl and Fries (loc. cit.) obtained good growth of *N. gossypii* and *Polyporus adustus* in mixed culture on a synthetic medium which would not support growth of either fungus alone. They suggested that *Nematospora* produced sufficient aneurin for growth of *Polyporus* (a view which was supported by their finding that *Nematospora* was largely independent of an external supply of this growth substance), and that *Polyporus* synthesized biotin and inositol thus making possible the development of *Nematospora*. Similarly Robbins and Kavanagh (1938a, b) demonstrated the synthesis of thiazole by certain fungi in a medium containing pyrimidine by growing *Phycomyces*, which requires both these substances, on the used medium. Schopfer (1938a, b) reports similar experiments.

Thus the effect of one organism, or of the medium in which it had grown, on a second whose growth substance requirements are known can be used to determine the nature of the growth substances synthesized by the first. A number of experiments on these lines was carried out.

1. *M. destruens* and *Phytophthora parasitica*.

Plates of medium A plus aneurin (4 γ per 100 c.c.) were inoculated with *M. destruens* and *P. parasitica*, the inocula being about 4 cm. apart (Pl. XXI, Fig. 6). Melanospora grew towards and surrounded the Phytophthora colony, but produced only short, malformed hyphae in the opposite direction. Perithecia were formed, these being most numerous in the neighbourhood of the Phytophthora colony. This arrangement of mycelium and perithecia of Melanospora suggests a response to some substance diffusing out from the colony of *P. parasitica*. Since *M. destruens* does not grow satisfactorily or produce perithecia on medium A plus aneurin in the absence of biotin, the observed response indicates that *P. parasitica* synthesizes biotin on this medium.

In another experiment liquid medium A and medium A plus aneurin (4 γ per 100 c.c.) were inoculated with *M. destruens* and *P. parasitica* together and separately. The results are given in Table VI. Growth of Melanospora in pure culture was negligible in either medium; that of Phytophthora in pure culture was negligible in medium A, but good in medium A plus aneurin. Both fungi grew slightly in mixed culture in medium A and grew well in mixed culture on medium A plus aneurin. In the last case numerous perithecia were formed by Melanospora. This experiment confirms the view that *P. parasitica* synthesizes biotin on a medium containing aneurin.

TABLE VI

Medium.	Inoculum.	Dry wt. mycelium (mg. per 100 c.c. medium) 12 days after inoculation.	Appearance, 12 days after inoculation.
A	<i>M. destruens</i>	negligible	—
"	<i>P. parasitica</i>	7	Growth very thin.
"	<i>P. cactorum</i>	negligible	—
"	<i>M. destruens</i> + <i>P. parasitica</i>	32	Growth thin,* Perithecia, none.
"	<i>M. destruens</i> + <i>P. cactorum</i>	29	Growth thin,* Perithecia, none.
A + aneurin	<i>M. destruens</i>	negligible	—
" "	<i>P. parasitica</i>	65	Growth good.
" "	<i>P. cactorum</i>	7	Growth very thin.
" "	<i>M. destruens</i> + <i>P. parasitica</i>	159	Growth thick,* Perithecia many.
" "	<i>M. destruens</i> + <i>P. cactorum</i>	130	Growth fairly thick,* Peri- thechia few.

* Mycelium was a mixture of hyphae of both species, not distinguishable macroscopically.

2. *M. destruens* and *P. cactorum*.

Similar experiments were carried out with mixed cultures of *M. destruens* and *P. cactorum*. The results were in general similar to those with *P. parasitica*, but the effects were less marked, since medium A is not favourable

to good growth of *P. cactorum* even with the addition of aneurin (v. Table VI, lines 3 and 8).

In plate cultures of medium A plus aneurin in which the inocula of the two fungi were about 4 cm. apart *Melanospora* grew towards and surrounded the *Phytophthora* colony. The latter remained small and was penetrated by the hyphae of the former. Numerous perithecia of *M. destruens* were formed over the surface of the *Phytophthora* colony and a few in the neighbouring region.

The two fungi were also grown in mixed culture in liquid media. The results, which resembled those with *P. parasitica*, are given in Table VI.

Thus *P. cactorum* resembles *P. parasitica* in that it synthesizes biotin in a medium containing aneurin.

3. *M. destruens* and *Phycomyces nitens*.

P. nitens resembles the species of *Phytophthora* in requiring an external supply of aneurin for good growth. It does not grow satisfactorily on medium A with or without aneurin since potassium nitrate is an unfavourable source of nitrogen. Table VII shows the behaviour of *Melanospora* and *Phycomyces* grown separately or together in Kögl and Fries' liquid medium (medium K in the table) without any addition and with (i) 10 γ aneurin per 100 c.c., (ii) an equivalent amount of pyrimidine, (iii) an equivalent amount of thiazole. Plus and minus strains of *Phycomyces* gave similar results.

TABLE VII

Medium.	Inoculum.	Dry wt. mycelium (mg. per 100 c.c. medium)	Appearance, 6 days after inoculation
K	<i>M. destruens</i>	negligible	—
"	<i>P. nitens</i>	42	Growth very thin
"	<i>M. destruens</i> + <i>P. nitens</i>	85	Growth thin*
K + aneurin	<i>M. destruens</i>	negligible	—
"	<i>P. nitens</i>	670	Growth good, sporangiophores many
"	<i>M. destruens</i> + <i>P. nitens</i>	787	Growth good,* sporangiophores many, perithecia numerous
K + pyrimidine	<i>M. destruens</i>	negligible	—
"	<i>P. nitens</i>	87	Growth thin
"	<i>M. destruens</i> + <i>P. nitens</i>	750	Growth good,* sporangiophores many, perithecia numerous
K + thiazole	<i>M. destruens</i>	negligible	—
"	<i>P. nitens</i>	80	Growth thin
"	<i>M. destruens</i> + <i>P. nitens</i>	92	Growth thin*

* Mycelium was a mixture of hyphae of both species, sporangiophores were those of *Phycomyces*; perithecia of *Melanospora*.

Table VII shows that the presence of *P. nitens* enables *M. destruens* to grow and produce perithecia on a synthetic medium containing aneurin which would otherwise be unsuitable. It is suggested that this is due to the production of biotin by the Phycomyces on a medium containing aneurin. The fact that these two fungi can grow in mixed culture on a synthetic medium with pyrimidine—on which neither grows satisfactorily in pure culture—suggests that Melanospora synthesizes thiazole in the presence of pyrimidine and biotin, the latter produced by Phycomyces. Conversely, Phycomyces is enabled to grow by obtaining thiazole from Melanospora.

4. *M. destruens* and *N. gossypii*.

TABLE VIII

Medium.	Inoculum.	Dry wt. mycelium (mg. per 100 c.c. medium)	7 days after inoculation.	Appearance, 7 days after inoculation
A	<i>M. destruens</i>	negligible		—
"	<i>N. gossypii</i>	"		—
"	<i>M. destruens</i> + <i>N. gossypii</i>	"		—
A+aneurin	<i>M. destruens</i>	"		—
"	<i>N. gossypii</i>	"		—
"	<i>M. destruens</i> + <i>N. gossypii</i>	"		—
A+biotin	<i>M. destruens</i>	24	Growth thin, peri- thecia none	—
"	<i>N. gossypii</i>	negligible		Growth fair,* peri- thecia few
"	<i>M. destruens</i> + <i>N. gossypii</i>	42		Growth good, peri- thecia many
A+aneurin+biotin	<i>M. destruens</i>	165		—
"	<i>N. gossypii</i>	negligible		Growth good,* peri- thecia many.
"	<i>M. destruens</i> + <i>N. gossypii</i>	173		

* Mycelium mostly that of Melanospora, slight growth of Nematospora.

Table VIII illustrates the results of experiments with mixed cultures of *M. destruens* and *N. gossypii* in various liquid media. These organisms did not grow either in pure or mixed culture on medium A or medium A plus aneurin (4 γ per 100 c.c.). Nematospora was also unable to grow on medium A plus biotin (4 γ per 100 c.c.) on which Melanospora grew but did not fruit. In mixed cultures on this medium Nematospora made some growth and a few perithecia of Melanospora were produced. This suggests that Nematospora synthesizes a small quantity of aneurin (which is in agreement with the results of Kögl and Fries, loc. cit.), and that Melanospora synthesizes a small quantity of inositol which is necessary for growth of the former. Medium A with the addition of biotin and aneurin gave good growth and

fruiting of Melanospora but negligible development of Nematospora. In mixed culture, however, *N. gossypii* showed appreciable growth, thus indicating synthesis of inositol by *M. destruens*.

5. *Growth of certain fungi on media previously used by M. destruens.*

Melanospora was grown on media A or K with the addition of biotin, on neither of which did it form perithecia. After one week the medium was filtered, the pH was adjusted to about 7.0 and fresh constituents of the original medium were added. After sterilization the used medium was inoculated with Phycomyces and the two species of Phytophthora. All three fungi showed increased growth on the used medium as compared with that on fresh synthetic medium, a result which indicates that some aneurin was present. Growth, however, was not so good as on fresh synthetic medium to which 4 γ aneurin per 100 c.c. had been added. Thus it is probable that *M. destruens* is able to synthesize a small quantity of aneurin in the presence of an adequate concentration of biotin but that the amount synthesized is insufficient for the production of perithecia.

In other experiments Melanospora was grown on medium A plus biotin and on medium A plus biotin plus aneurin. After one week the media were filtered, sterilized, and inoculated with *N. gossypii*. Growth of the latter was good, indicating that a certain amount of inositol had been produced by *M. destruens*.

VII. DISCUSSION

The experiments described above show that *M. destruens* requires biotin for mycelial growth and does not produce perithecia in the absence of aneurin (vitamin B₁) or of its pyrimidine component. Experiments with mixed cultures have indicated that this fungus synthesizes inositol and a small amount of aneurin (insufficient for formation of perithecia) in the presence of biotin. In the presence of both biotin and the pyrimidine component of aneurin the thiazole component is synthesized.

The observation that Melanospora requires biotin for mycelial growth and aneurin for production of perithecia is of theoretical interest as indicating a difference in the functions of these two growth substances. When both are present the concentration of either influences growth and fruiting. The special relationship of aneurin to fruiting is further shown by the fact that with increasing concentration it raises the glucose concentration optimal for fruiting, an effect which is not shown by biotin. So far as they have been studied, *Podospora curvula* and two species of *Sordaria* behave similarly to Melanospora.

Biotin, aneurin, and inositol have been shown by various investigators to be of importance for the growth of a number of fungi, their relative importance varying with the species. Some fungi are able to grow and produce spores in the absence of an external supply of these substances, others require

an external supply of one but not of the others, while yet others require two or even all three. It is probable that all fungi require all three growth substances but that some synthesize their own supply from a synthetic medium. That this is the case is supported by the numerous reports of the 'stimulatory' effect of one fungus on another.

Occasionally certain organisms have been described which need an external supply of yet other substances for satisfactory development. Thus β -alanine was shown by Williams and Rohrman (1936) to be necessary for maximum growth of some yeasts and by Buston et al. (1938) to be of importance in the nitrogen metabolism of *N. gossypii*. L-leucine was shown by Miller (1935) to increase yeast crops. Very little is known as yet about the functions of these substances, but it is possible that the majority of fungi are able to synthesize them and hence only a few examples of organisms requiring an external supply have been described.

As pointed out by Kögl (loc. cit.) and confirmed in this work, biotin and aneurin produce striking effects at very low concentrations, in which respect they differ from inositol. Thus they resemble the auxins of higher plants, with which, however, they have no chemical relationship. Auxins have given negative results when tested on a wide range of fungi (Ronsdorf, 1935, and others), although Itzerott (1938) has recently described slight stimulation of *Ustilago Zeae* by solutions of 3-indole-acetic acid.

Schopfer (1938a, b) and Robbins (1937) have divided the organisms studied by them into groups according to their growth-substance requirements. In view of the somewhat different requirements shown by saltant strains of *Melanospora* it would seem doubtful if any rigid physiological classification of such a kind is possible.

Knowledge of the growth-substance requirements of certain fungi can be used as a means of testing for the presence of these substances and to a certain extent of estimating the amount present. The test organism, however, must be standardized against pure preparations of the growth factors before use; otherwise there is considerable risk of an unrecognized change in the synthesizing powers of the organism rendering the test invalid. A positive result obtained with a suitably standardized test organism would indicate the presence of a particular growth substance, but the converse would not necessarily be true. Negative results would require further analysis, i.e. the proof that failure of growth was not due to lack of some other growth substance or of any of the ordinary foodstuffs, presence of inhibitory substances, &c.

If the above precautions are observed the following scheme may be suggested as providing suitable qualitative tests.

(1) *Aneurin*. A positive result obtained with species of *Phycomyces* (*P. nitens* or *P. Blakesleeanus*), *Phytophthora* (*P. parasitica*, *P. cactorum*, &c.), or certain Basidiomycetes would indicate the presence of aneurin or of its thiazole and pyrimidine components. The exact reaction of the test organism

to aneurin, a mixture of thiazole and pyrimidine, or to either alone must be determined to permit a distinction between these to be made. Thus the strains of *Phytophthora* used in the present investigation react only to the aneurin molecule as such while *Phycomyces* reacts also to a mixture of the components.

Similarly the presence of aneurin or of both components or of pyrimidine is demonstrated if *M. destruens* or a fungus with similar requirements produces perithecia.

(2) *Biotin*. If *Melanospora* grows on a given medium, or if *Nematospora* grows on the medium with or without the addition of inositol, biotin is present.

(3) *Inositol*. This substance is present in a medium if *Nematospora* is able to grow satisfactorily on it with or without the addition of biotin or of some inositol-free source of this substance (such as lentil extract from which the inositol has been removed by precipitation by barium hydroxide (Buston and Pramanik, loc. cit.)).

A quantitative method of estimating vitamin B₁ with the aid of a fungus would be very valuable since fungi react to much smaller concentrations than do the test animals which are generally used. Numerous investigators have attempted to use yeast for this purpose. Schopfer and Jung (1937) have elaborated a method of using *P. Blakesleeanus* and have obtained good agreement between tests made with this fungus and with animals. This method is, however, open to some objections. It was pointed out by Schopfer and Jung that *Phycomyces* is able to use the thiazole and pyrimidine components of aneurin so that a positive result does not necessarily measure the amount of aneurin present. This is a fact of some importance, since the components are not used by the usual test animals. Fungi, such as the strains of *Phytophthora* studied in the present investigation, which need the aneurin molecule as such would be more suitable than *Phycomyces* for this purpose. More recently Bonner and Erickson (1938) have pointed out the desirability of a further knowledge of the range of thiazole and pyrimidine compounds which this fungus uses in order to assess its value as a test organism.

A further difficulty in the estimation of a growth substance by the use of fungi is the possibility of interference through the introduction into the medium of foodstuffs, other growth factors, or inhibitory substances in association with the growth substance to be estimated. Schopfer and Jung demonstrated that the response of *Phycomyces* to a particular dose of aneurin is influenced by the amount of asparagin present. They were unable to use the method for the estimation of aneurin in milk until the latter had been freed from casein. Similarly the marked influence of glucose concentration on the number of perithecia produced by *Melanospora* limits the usefulness of the latter as a means of estimating aneurin.

Where the growth substance to be estimated is present in relatively high concentration and is unaccompanied by an excessive quantity of foodstuffs the problem is not so complex. Small quantities of the test solution could

then be added to a medium known to be suitable in every other respect for growth of the test organism and the effects compared with those produced by the addition of known quantities of pure growth substance to the same basal medium. When, however, small quantities of growth substance are associated with high concentrations of foodstuffs, it would be desirable to remove the latter beforehand. This is not always practicable, e.g. with carbohydrates. Alternatively the influence of different concentrations of various food substances on the response to growth substance would require to be determined.

The possibility of the presence of inhibitory substances in the mixture to be tested must not be overlooked and as far as possible adjustments should be made to negative these when present.

If attention be paid to these possible sources of error and their effects be removed as far as possible by a suitable choice of the conditions of the test, reasonably accurate estimations of growth substance concentration may be attained by the use of fungi.

It has already been pointed out above that aneurin can be most conveniently estimated by the use of a fungus which requires the vitamin molecule as such. The amount of biotin in a given medium might be estimated by measuring the amount of growth of *Nematospora* in the presence of excess inositol and aneurin and with due regard to suitable food supply, &c. Similarly inositol might be estimated by the growth of *Nematospora* in the presence of excess biotin and aneurin. These would be conveniently added in the form of lentil extract freed from inositol by precipitation with barium hydroxide. In each case the method would have to be standardized by precise experiments with known quantities of the growth substances to be estimated.

VIII. SUMMARY

1. *Melanospora destruens* does not grow on a synthetic medium consisting of glucose, potassium nitrate, and salts (medium A) or on medium A with the addition of aneurin (vitamin B₁). It grows but does not fruit on medium A with the addition of Kögl's biotin. The further addition of aneurin increases the growth and causes the production of numerous perithecia.

2. Aneurin can be replaced by a mixture of its thiazole and pyrimidine components or by the latter alone.

3. Inositol has no effect on growth or fruiting.

4. Certain saltant strains of *M. destruens* were able to grow and fruit in the absence of biotin or aneurin, but fruiting was increased by the addition of either of these and still further increased by the addition of both. It is suggested that changes in the growth-substance requirements of the stock strain may explain the difference between a previous result and the present ones with aneurin.

5. Biotin and aneurin are effective in very low concentration. Mycelial growth and production of perithecia are influenced by increasing concentra-

tion of either or both substances until a point is reached at which other factors are probably limiting.

6. Increase in aneurin concentration raises the percentage glucose optimal for perithecial production. Biotin has no such effect.

7. The findings of other investigators with respect to the growth-substance requirements of *Nematospora gossypii* and species of *Phycomyces* and *Phytophthora* are in general confirmed.

8. *Podospora curvula*, *Sordaria* sp., and *S. fimicola* resemble *M. destruens* in their growth-substance requirements.

9. The synthesis of one or more growth substances by certain fungi was demonstrated by means of mixed cultures or by growing one fungus on media staled by another.

10. The results are discussed and the possibility of using certain fungi as a means of testing for the presence of and estimating the amount of these growth substances is considered.

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EXPLANATION OF PLATE XXI

Illustrating Dr. L. E. Hawker's paper on 'The Nature of the Accessory Growth Factors influencing Growth and Fruiting of *Melanospora destruens* Shear., and of some other Fungi'.

Perithecia show as small black objects which can be clearly seen with the aid of a hand lens. Diameter of Petri dishes 9 cm. All cultures 10 days old.

Fig. 1. *Melanospora destruens*, on medium A plus aneurin (4 γ per 100 c.c. medium). Illuminated from rear to show limits of colony (m.c.) and irregular growth. The dark objects are irregular knots of hyphae. No perithecia formed.

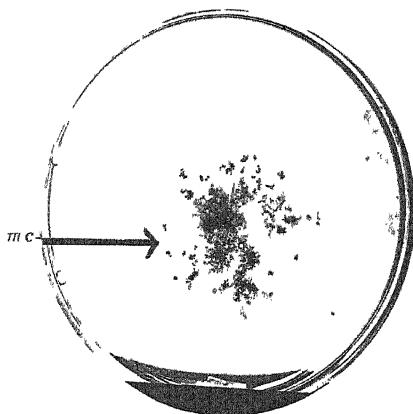
Fig. 2. *M. destruens*, on medium A plus biotin (4 γ per 100 c.c. medium). The hyphae have formed a thin layer over the surface of the medium. No perithecia formed.

Fig. 3. *M. destruens*, on medium A plus biotin plus aneurin. Numerous perithecia formed.

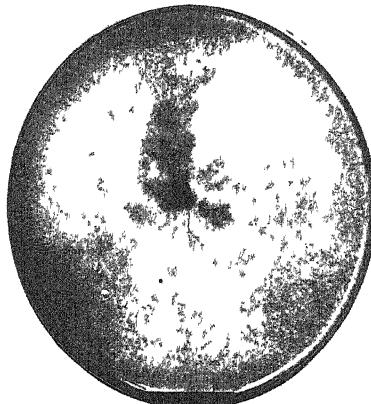
Fig. 4. *Phytophthora parasitica* on medium A. Colony small and thin. m.c. = margin of colony.

Fig. 5. *P. parasitica*, on medium A plus aneurin (4 γ per 100 c.c. medium). Colony large with thick mass of white aerial hyphae.

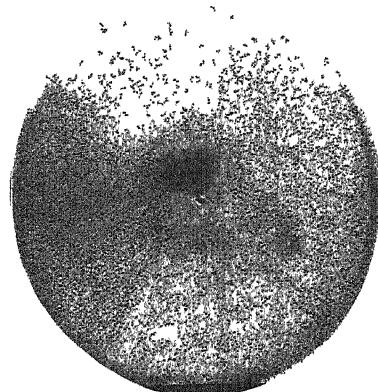
Fig. 6. *M. destruens* and *P. parasitica* in mixed culture on medium A plus aneurin. P = inoculum of *Phytophthora*, M = inoculum of *Melanospora*. Note ring of perithecia surrounding the *Phytophthora* colony.



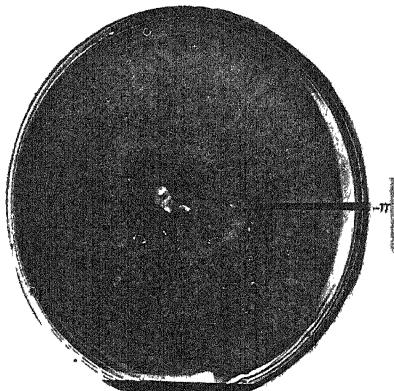
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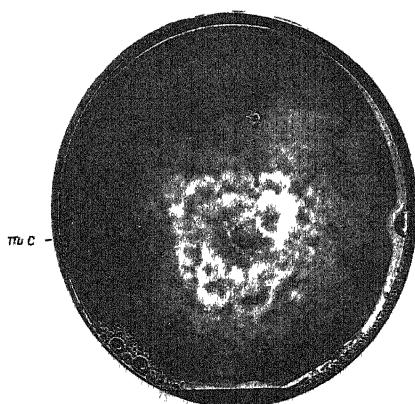
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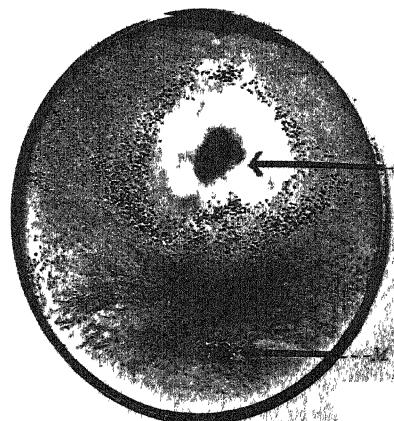
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Acid Metabolism and Respiration in Succulent Compositae

I. Malic Acid and Respiration during Starvation in *Kleinia articulata*

BY

D. THODAY

AND

K. MAIRGRETTE JONES¹

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With seven Figures in the Text

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INTRODUCTION

IT has been shown in previous papers on *Kleinia articulata* (Thoday and Evans, 1932, 1933) that the stem, in full vigour, shows the acid periodicity characteristic of other succulents. Malic acid is the chief acid present. Traces of oxalic acid were found in the outermost tissues of young plants, but this is soon deposited in the form of calcium oxalate crystals in the hypodermis. The periodicity therefore is one of malate content.

It was found that the malic acid was to a large extent associated with calcium, and that calcium malate showed very marked and sharp localization in the pith and in certain parts of the inner cortex. With increasing age, calcium accumulates in the cells of the pith, but the cortical regions diminish in size until no calcium remains. The diurnal changes in titratable acidity, however, occur mainly in the cortex: the pith shows little, if any, change. The sharp delimitation of the accumulated calcium malate seemed to offer the possibility of obtaining evidence as to its functional significance.

Jackson and Thoday (1939; see also M. W. P. Roberts, 1933) made observations on the sprouting of branches, by microchemical methods, but found

¹ This paper embodies the substance of part of a thesis presented by the second author for the degree of Ph.D. in the University of Wales.

no evidence that calcium malate was drawn upon. Preliminary observations recorded on starved stems suggested, however, that further work on starvation and respiration might be helpful.

The previous work had emphasized the great variation in the material. On the other hand, the sharp localization of calcium malate suggested the desirability of experiments with excised pith and other tissues separately. Both considerations pointed to the use of small portions of material, and for this purpose suitable micro-methods had to be adopted.

Malic acid content.

METHODS

For total malate content the lead precipitation method outlined by Bennett-Clark and Woodruff (1935) was employed. Basic lead acetate was replaced by tribasic lead acetate prepared according to the directions given by Hartmann and Hillig (1932). The technique was standardized throughout, all analyses were carried out in duplicate using a different volume of extract for the two determinations, and the mean value was taken to represent the 'malic acid' content.

The material was pulverized in a mortar, using clean silver sand and distilled water, and the extract was then filtered through a pad of asbestos wool, with the aid of a suction pump. An aliquot part of the water extract was placed in a centrifuge tube, neutralized to pH 7, and a drop of capryl alcohol added. The tribasic lead acetate solution was added drop by drop from a 1-c.c. micro-burette with a fine nozzle. Towards the end of the addition the precipitate was centrifuged out after the addition of each drop. When precipitation was complete the supernatant liquid was siphoned out, and the precipitate washed and re-centrifuged twice. The washed precipitate was then suspended in water, decomposed with H₂S, and the lead sulphide centrifuged out. The supernatant liquid was decanted into a small beaker, together with two washings of the lead sulphide. Excess H₂S was driven off by gentle boiling and the use of lead acetate paper. The acid was titrated against baryta (approx. N/50), using bromthymol blue as indicator.

Results of preliminary tests on *K. articulata* stem extract with a known amount of malic acid solution added are given in Table I.

TABLE I
Baryta, 1·02 × N/50. Malic acid solution, 0·9702 gr. in 100 c.c.

	Baryta required. (c.c.)	Mean value.	Malic acid. (mg.)
1. 5 c.c. extract + 0·2 c.c. solution	. . 5·95	6·00	8·22
2. " + 0·2 "	. . 6·06		
3. 5 c.c. extract only	. . 4·56	4·50	6·17
4. " "	. . 4·45		
Malic acid added	1·94 mg.
Malic acid recovered	2·05 "

Carbon dioxide output.

For the determination of CO₂-output a micro-absorption tube was devised. It was necessary to dilute and reduce the volume of baryta used, at the same time ensuring complete absorption of the carbon dioxide. It was envisaged that an absorbing tube of capillary dimensions would be possible if a by-pass were provided for the circulation of the baryta. After preliminary trials the micro-absorption tube shown in Fig. 1 was constructed. (The method of circulation has been similarly applied in the conductivity cell described by Newton, 1935.) The reservoir portion is made of glass tubing of 1.5 cm. internal diameter, and the spiral part of capillary tubing of 1.5 mm. bore. The lateral position of the by-pass is determined by the connexion of the spiral to the bottom of the reservoir, which is dictated by the technique of titration (see below). The volume of baryta used is 6 c.c.; absorption is efficient when using N/50 strength, and there is no clogging due to the deposited carbonate.

A battery of these tubes was arranged round a Blackman air-current commutator¹ taking two sets of eight tubes, and thus allowing of two simultaneous experiments. The rate of the air current was adjusted at approximately 100 c.c. per hour by means of a needle-valve of the form described by Knipp (1928), included in the circuit between the plant chamber and the commutator. For experiments at higher temperatures (25° C.) the plant chambers were enclosed in an air thermostat with double walls, the space between being filled with light magnesium oxide lagging. The chamber was electrically heated by 'Glowray' wire wound round a mica frame placed on the floor of the chamber, with mercury-toluol control. The temperature as registered by a thermometer placed in front of the toluol bulb fluctuates as the current through the heating element goes on and off, showing a maximum range of about 1° C. No fluctuation is, however, observable with a thermometer inside the glass plant chambers.

The titration of the baryta is carried out in the absorption tube, a current of CO₂-free air being used for stirring. Near the end-point, the rate of the air current is increased, and the amount of acid added is carefully controlled, very small drops being swept off the tip of the micro-burette. In order to allow for any slight contamination with atmospheric CO₂ that may occur during the filling of the tubes and their subsequent removal to the titration circuit, blank determinations were carried out under similar conditions, these values being employed in the calculations.

Oxygen intake.

For oxygen intake determinations a constant-pressure type of manometric

¹ Procured with a special grant from the University of Wales.

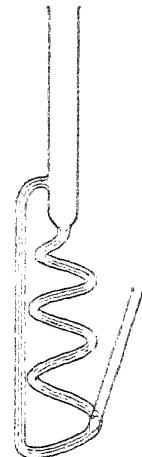


FIG. 1. Micro-absorption tube.

apparatus similar to that described by Thoday (1932) was used in the earlier work, and later a modification of Dixon's apparatus (Dixon, 1934). For the latter, flasks suitable for the material under investigation were specially obtained, which like the usual type were supplied with standard ground joints. Difficulty was experienced at first in using this apparatus, unexpected volume changes being sometimes recorded by the manometer. After extensive trials the source of error was located in the grinding of the flasks and joints. The fit of the standard joints was not sufficiently accurate, and a gradual movement of the flasks on their joints could occur. Selected flasks were therefore reground on to each joint, numbered, and no longer regarded as interchangeable. This procedure eliminated the erratic behaviour previously encountered. Electrically heated water-baths were employed for higher temperatures. In these an electric immersion heater as described by Leach (1931) but of Pyrex tubing, and a mercury-toluol thermo-regulator were used.

RESULTS: *KLEINIA ARTICULATA* HAW.

Total Malate

Borgström (1934) has brought forward evidence for the presence of citric acid in the leaves of many species of *Kleinia*, and in the stem of *Kleinia pendula* D.C. In view of this, the nature of the acid present in *Kleinia articulata* was again investigated. Extracts from old and young stems, and from leaves, were examined. The addition of calcium chloride to the neutral extract gave no precipitate even after boiling and allowing to stand for several hours, conditions which would precipitate any citrate present. This confirms the earlier work of Evans (1931).

Quantitative work on the total malate content indicates a similar trend to the results on titratable acidity given by Evans (1931). Table II shows that

TABLE II

Material.	Fresh wt. (gm.)	Malic acid (gm. per 100 gm. fresh wt.)		Mg. equiv.
		Stems	Leaves	
Sept. 19, 10 a.m.				
i. Six young shoots, 1 cm. long				
Stems	0.475	0.51	7.6	
Leaves	5.21	0.30	4.5	
Sept. 20, 10 a.m.				
2. Six shoots, 1.5 cm. long				
Stems	3.01	0.77	11.5	
Leaves	20.11	0.33	4.9	
Oct. 26, 10 a.m.				
3. Single shoot, 12.5 cm. long, bearing an inflorescence in bud, and a small axillary shoot				
Small axillary shoot (including leaves) . .	0.365	0.18	2.7	
Tip of stem, with inflorescence . .	0.61	0.35	5.2	
Middle portion of stem	1.74	0.58	8.7	
Basal portion of stem	2.49	0.81	12.1	
Three large leaves, 7-7.5 cm. long . . .	2.69	0.25	3.7	

the malic acid content of the stem increases with age, and in the main stem increases from tip to base. The lower acid content of leaves is also shown.

Thoday and Evans (1933) made a rough estimate of total malate in stems of *Kleinia articulata* from the balance-sheet of the principal ions. It will be observed that their value, 10 mg. equivalents, is of the same order as the values given in Table II, which range from 7.6 to 12.1 for samples of stem. (Rather higher average values are indicated in Table III.)

Pith v. remainder.

Table III gives the results of separate acidity determinations on the colourless pith and the remaining tissue, carefully separated with a scalpel, and leaves, at three different times of day. The mean values are summarized in Table IV.

TABLE III

Total Malic Acid (gm. per 100 gm. fresh weight)

10 a.m.	Leaves.	Stem.	
		Remainder.	Pith.
Sept. 21	—	1.04	0.84
" 22	0.67	1.44	0.80
" 23	0.51	1.60	0.89
Oct. 12	0.66	1.28	0.87
" 14	—	0.99	0.71
" 30	0.46	1.19	0.89
Mean	0.58	1.26	0.83
S.D. of mean	±0.05	±0.10	±0.03
 3 p.m.			
Nov. 20	0.37	0.88	0.82
" 26	0.37	0.79	0.67
" 27	0.34	0.87	0.74
" 28	—	0.91	0.94
Dec. 2	—	0.89	0.80
Mean	0.36	0.87	0.79
S.D. of mean	±0.01	±0.02	±0.05
 8.30 p.m.			
Dec. 10	0.34	0.99	0.96
" 12	0.30	0.82	0.86
" 15	0.26	0.73	0.80
Mean	0.30	0.85	0.87
S.D. of mean	±0.02	±0.08	±0.05

TABLE IV

Total Malic Acid. Summary of Mean Values

	Leaves.	Stem.	
		Remainder.	Pith.
10 a.m. Sept. 21-Oct. 30	. . .	0.58 ± 0.05	1.26 ± 0.10
3 p.m. Nov. 20-Dec. 2	. . .	0.36 ± 0.01	0.87 ± 0.02
8.30 p.m. Dec. 10-15	. . .	0.30 ± 0.02	0.85 ± 0.08
Means as mg. equivalents	. . .	8.6 ± 0.7	18.8 ± 1.4
		5.4 ± 0.1	13.0 ± 0.3
		4.5 ± 0.3	12.7 ± 1.1

A diurnal variation in the total acid content of the leaves and the outer tissue of the stem corresponding with the diurnal periodicity in titratable acidity is evident. Such a marked fluctuation is not shown by the acid values for the pith.

In view of the consecutive nature of the experiments, there is a possibility of a 'seasonal progression' being superimposed on the diurnal fluctuation, but the pith values give no indication of such a change and its effect, if any, is probably unimportant.

The average values for the fall in total acidity between morning and afternoon, and comparable values for titratable acidity (Thoday and Evans, 1932) were as follows:

	Mg. equivalents per 100 gm.	Outer tissues.	Pith.
Total malate: Sept.-Dec. 1935, 10 a.m. to 3 p.m.	.	5·8	0·6
Titratable acidity: June 1930, 10.30 a.m. to 5 p.m.	.	3·2	0·2

The agreement is reasonably close, considering the differences in date and conditions of growth, and the later start in June relative to sunrise.

Malic Acid and Calcium

Outer cortex. It has been shown (Thoday and Evans, 1932) that in the green outer cortex of *K. articulata* no calcium is precipitated by alcoholic oxalic acid; but as potassium is abundant in this zone it was inferred that malic acid occurs in these assimilatory cells chiefly as the potassium salts. The evidence of its presence, however, was mainly indirect and not as satisfactory as in the case of calcium malate.

The next series of experiments was therefore carried out in order to obtain some quantitative information regarding the acid content of this assimilatory zone. The stem was divided into outer cortex and the remaining tissue. The latter includes (as far as possible) the calcium-containing cells of the inner cortex as well as the pith, leaving as outer cortex a zone with minimum calcium content. Simultaneous determinations of calcium and 'malic acid' content were made, to see how closely the two are linked up in the different tissues. The calcium was determined by precipitation with ammonium oxalate, solution of the precipitate by dilute H_2SO_4 and titration against $KMnO_4$. The data are given in Table V, expressed in mg. equivs. in order to facilitate comparison of the malic acid and calcium figures.

Each record shows a higher total acid value for the remaining tissue than for the outer cortex. In the former, the amount of acid present and the calcium content are closely related. On the other hand, in the outer cortex, although the separation of the non-calciferous zone from the calciferous inner cortex has been incomplete, as was inevitable, the acid is definitely in excess of the much lower concentration of calcium present, in every case. The

TABLE V

*Malic Acid and Soluble Calcium in Outer Cortex and Remainder of Stem
(mg. equivalents per 100 gm. fresh weight). Stems of Current Season
weighing 8-12½ gm.*

Date, 1935.	Outer cortex.		Remainder.	
	Malic acid.	Calcium.	Malic acid.	Calcium.
Nov. 6	17·9	8·3	18·3
" 7	10·6	8·2	12·8
" 8	10·7	5·8	13·4
" 11	9·1	5·3	15·1
" 12	10·0	5·8	18·3
" 14	9·1	4·2	18·0
Average	11·23	6·27	16·0
Ca % of malate	55·8%		97%

TABLE VI

Date, 1935.	Malic acid minus calcium (mg. equivs.).	
	Outer cortex.	Remaining tissue.
Nov. 6	+9·6
" 7	+2·4
" 8	+4·9
" 11	+3·8
" 12	+4·2
" 14	+4·9
Mean value \pm S.D. of mean	+5·0 \pm 1·0
		+0·5 \pm 0·7

differences between the 'malic acid' and calcium content in the two portions are set out in Table VI.

The presence of malic acid in the outer cortex may therefore be regarded as established. As microchemical tests have already shown that calcium is not detectable in the sap, the acid must be associated with potassium and sodium (Thoday and Evans, 1933, pp. 17-18).

The outer cortex in these analyses averaged 21·8 per cent. of the total fresh weight. The average malate content for the whole stem is therefore 21·8 per cent. of 11·23 + 88·2 per cent. of 16·0, which is 15·0 mg. equivs. A corresponding calculation from the data in Table III, where the pith was separated, gives 15·6 mg. equivs. These two batches of material may therefore be regarded as rather closely comparable. Since in each case the remainder has the higher malate content, it follows that the region common to the two remainders is richest in malate. This includes the inner cortex, bundle zone, and more or less of the outermost calciferous pith. In stems of these ages both inner cortex and outer pith give an abundant calcium malate precipitate in alcohol. Microchemical and analytical data are thus concordant.

Leaves.

Leaves were available from the material used for the starvation experiments to be described in the next section. These were employed for estimating the

'malic' acid and calcium content of the laminae and petioles separately. The results are given in Table VII.

TABLE VII

Date, 1935.	Mg. equivs. per 100 gm. fresh weight.			
	Laminae.	Petioles.	Laminae.	Petioles.
1. Sept. 26	10·2	8·5	3·6	4·3
2. " 30	8·4	5·5	3·6	6·1
3. Oct. 7	9·4	5·4	6·4	10·7
4. " 7	8·1	5·4	5·8	10·5

The acid content of the laminae is greater than that of the petioles while the reverse is true for calcium. These results may be compared with the higher acid content of the outer tissues as compared with the pith of the stem, and the higher calcium content of the pith.

Malate and Calcium during Starvation

Quantitative estimations of the calcium and 'malic' acid content were now carried out before and after starvation in the dark. All the material had been grown under the same conditions, and the shoots chosen for a particular experiment were similar in external appearance. The experiments were successive so that the shoots within one experimental lot were more comparable one with the other than were the shoots of one experiment with those of another experiment.

The problem of minimizing sampling errors was important as one lot of material had to be analysed at the start and another at the end of the starvation period. In *K. articulata* variation in acid content from tip to base in the individual stem has to be allowed for as well as variation from stem to stem. Bearing this in mind, the choice of material was accomplished as follows:

The joints were divided into two lots (A and B), and the individual joints then divided into three portions of approximately equal size (A₁, 2, 3; B₄, 5, 6). The apical and basal segments of lot A and middle segments of lot B (i.e. 1, 3, 5) were taken for the initial analyses, and the apical and basal segments of lot B, and middle segments of A (i.e. 2, 4, 6) for the final analyses. Lots (2), (4), and (6) had their cut ends waxed, and were placed horizontally on damp sand and left in the dark.

The calcium and 'malic' acid contents of the water extract are expressed throughout as mg. equivs. per 100 gm. fresh weight, and values after starvation are bracketed. Results obtained in a test for which the material was chosen in a similar way and divided into the six lots, all of which were analysed immediately, are included in Table VIII A with the data for the first experiment detailed here.

Expt. 29. Sept. 26th-Oct. 2nd, 1935.

Eight stems were used, the average size being 6·5 by 0·9 cm. The starved segments were analysed after 6 days in the incubator at 30° C. They showed

injection of the outer pith, the order of increasing degree being (4), (2), (6). Injection was externally visible to some extent in (4) and (2).

TABLE VIII A

	Experiment 29.			Sampling test.	
	Malic acid.	Calcium.	Malic acid.	Calcium.	
(1)	.	.	14.6	12.4	11.0
	.	.	(12.1)	(9.2)	11.7
Lot A	3	.	20.9	12.6	16.8
	(4)	.	(10.6)	(6.2)	12.0
Lot B	5	.	16.0	8.9	11.8
	(6)	.	(14.9)	(9.9)	15.2

The data can be analysed in two ways:

1. *Lots A and B are considered separately*, the mean values for the apical and basal portions being compared with the values for the middle portions of the same lot.
2. *Lots A and B are considered together*. The segments of one lot are taken to be comparable with the corresponding segments of the other lot. For the whole stem 1+5+3 is taken to represent the initial condition, and (2)+(4)+(6) the final condition.

TABLE VIII B

Analysis by Method 1

		Experiment 29.		Sampling test.	
		Malic acid.	Calcium.	Malic acid.	Calcium.
A.	$\frac{1+3}{2}$	(Initial)	17.7	12.5	13.9
	(2)	(Final)	(12.1)	(9.2)	11.7
		Loss	+5.6	+3.3	+2.2
B.	5	(Initial)	16.0	8.9	11.8
	$\frac{(4)+(6)}{2}$	(Final)	(12.7)	(8.0)	13.6
		Loss	+3.3	+0.9	-1.8

TABLE VIII C

Analysis by Method 2

		Experiment 29.		Sampling test.	
		Malic acid.	Calcium.	Malic acid.	Calcium.
	$\frac{1+5+3}{3}$	(Initial)	17.2	11.3	13.2
	$\frac{(4)+(2)+(6)}{3}$	(Final)	(12.5)	(8.4)	13.0
		Loss	+4.7	+2.9	+0.2

The sampling test data indicate that the second method of treatment is likely to give better elimination of the sampling errors, the differences between different parts of the same shoot being evidently a greater source of error than the differences between paired lots. This is due mainly to the greater accumulation of acid and calcium in the basal segments, particularly at this early part of the growing season. The first method of calculation gives two measures of loss which are weighted in opposite senses by this factor. Increasing the number of values is, however, a gain in estimating the error of mean values. Therefore, in a subsequent series of experiments, in order to make the most of the data statistically, losses have been calculated from the corresponding A and B values, for apical, middle, and basal segments separately.

Before dealing with these, two other experiments should be mentioned. In Expt. 30, parallel with Expt. 29 but a few days later, the period of starvation was 10 days (Sept. 30–Oct. 10). The average loss of malate was 9.3 mg. equivs, as compared with 4.6 mg. equivs in 6 days.

In Expt. 33, twelve smaller, younger stems were used, and the period of starvation was 13 days (Oct. 18–31). The average loss was 11.6 mg. equivs. These three results suggested that 'malic' acid diminishes early and progressively.

In the other experiments, seven in all, the material was starved at 30° C. for 13 days. The malate and calcium data are given in Table IX, together with the mean content for corresponding portions from all seven experiments, with their standard deviations. The losses for each segment are set out in Table X with further statistical data. The initial malate values (Table IX) show a fair degree of uniformity. The variance is much greater for the starved segments, which indicates variability of behaviour during starvation. This is shown even within the same experiment, between one segment and another. This applies particularly to the basal segments. The correlation coefficients of malate losses between different segments in the same experiment are as follows:

Tip with middle	0.634
Tip with base	0.304
Middle with base	0.321

For a probability of 19:1 against a coefficient being fortuitous, based on seven pairs of data, the limit is 0.6664; so that even the correlation between tips and middles is not quite statistically significant. The other two coefficients are far below even the 9:1 probability limit (0.5822).

In striking contrast with this irregularity of behaviour is the high degree of correlation between the individual malate and calcium losses. Calculated from the twenty-one pairs of data, on the basis of the deviations from their respective means, the correlation coefficient is 0.827. As the 19:1 limit is 0.413 in this case, the correlation is highly significant.

TABLE IX

Malic Acid and Soluble Calcium Content (mg. equivalents per 100 gm. fresh weight) before and after Starvation in the Dark at 30° C. for 13 Days (values for starved segments are bracketed)

Expt. No.	.	31	32	34	35	36	37	38	Mean and S.D. of mean
Dates, 1935-6	.	Oct. 7-20	Oct. 15-28	Nov. 4-17	Nov. 5-18	Dec. 4-17	Dec. 9-22	Jan. 23- Feb. 5	
<i>Malic acid</i>									
A	1	.	.	13.9 (7.8)	10.3 (4.2)	12.4 (4.0)	11.7 (1.4)	13.7 (13.9)	11.4 (7.1)
	(2)	.	.						10.6 (9.8) (6.89±1.57)
B	3	.	.	13.4 (3.0)	14.9 (3.4)	14.9 (7.6)	13.9 (2.2)	17.0 (11.9)	15.0 (4.4)
	(4)	.	.						14.1 (8.4) (5.84±1.34)
	5	.	.	14.6 (12.1)	11.9 (6.7)	11.5 (10.5)	11.4 (1.2)	15.5 (12.9)	10.5 (4.9)
	(6)	.	.						14.7 (12.3) (8.66±1.69)
<i>Calcium</i>									
A	1	.	.	13.6 (11.5)	14.2 (6.7)	10.8 (4.8)	11.3 (1.3)	8.6 (11.3)	10.9 (6.6)
	(2)	.	.						7.3 (7.9) (7.16±1.35)
B	3	.	.	18.0 (6.0)	14.7 (5.5)	14.2 (9.9)	13.4 (3.0)	13.8 (9.4)	9.0 (3.3) (5.3)
	(4)	.	.						12.4 (6.06±1.02)
	5	.	.	14.7 (13.7)	12.0 (10.5)	10.7 (13.1)	14.2 (2.2)	13.4 (12.7)	15.4 (5.1) (12.8)
	(6)	.	.						11.5 (13.13±0.66) (10.01±1.71)

TABLE X

Malic Acid Losses

Expt. No.	.	31	32	34	35	36	37	38	Mean and S.D. of mean
Tip, A-B	.	10.9	6.9	4.8	9.5	1.8	7.0	2.2	6.16±1.3
Middle, B-A	.	6.8	7.7	7.5	10.0	1.6	3.4	4.9	5.99±1.09
Base, A-B	.	1.3	8.2	4.4	12.7	4.1	10.1	1.8	6.09±1.71
Means:									
$\frac{(1+3+5)-(2+4+6)}{3}$		6.3	7.6	5.5	10.7	2.5	6.8	3.0	6.07±0.76

General average of 21 differences 6.08 ± 0.76 (S.D. calculated from the 21 differences).

Calcium Losses

Expt. No.	.	31	32	34	35	36	37	38	Mean and S.D. of mean
Tip, A-B	.	7.6	8.7	0.9	8.3	-0.8	7.6	2.0	4.90±1.52
Middle, B-A	.	3.2	5.3	5.9	12.9	2.1	8.8	3.6	5.99±1.42
Base, A-B	.	4.3	4.2	1.1	11.2	1.1	3.9	-0.4	3.63±1.44
Means:									
$\frac{(1+3+5)-(2+4+6)}{3}$		5.0	6.1	2.6	10.8	0.8	6.8	1.7	4.84±0.80

General average of 21 differences 4.84 ± 0.80 (S.D. calculated from the 21 differences).

The general averages themselves are fully significant. The average losses from different segments are also all significant. It is therefore clear that during starvation at 30° C. for 13 days both malate and soluble calcium disappear from all parts of the stem of *K. articulata*, from apex to base, and that the disappearance of calcium and malate are closely correlated.

It was conceivable that some calcium might leach out into the sand on which the starving segments lay, though this was not very likely in view of the thickness of the cuticle and the waxing of the cut ends. Experiments with pieces suspended in damp air so that no leaching was possible confirmed the loss of calcium.

An attempt was made to demonstrate the deposition of calcium *in situ* by analysing the residue after extraction. The supply of material, however, had by that time been much reduced and the results were inconclusive.

It may nevertheless be inferred from the close correlation between the calcium and malate losses that the bulk, if not all, of the calcium lost from the sap is precipitated in insoluble form, probably as carbonate.

Experiments with single shoots made the following June and July showed greater uniformity of material in the resting season, and smaller malate loss. Five experiments at 30° C. of much longer duration, viz. 22–27 days instead of 13, gave a mean loss of 3.3 mg. equivs. (Mean initial content, average of tip and base, 12.6 mg. equivs.; S.D. of mean 0.17.) Only the outer pith had become injected.

Experiments at 25° C. a little earlier in the year also indicated a deferring of the use of malate; for the two shoots after 16 days' starvation, in March, showed injection only in the outermost pith and losses which were not significant. Four similar experiments in April and May lasting 23–30 days gave average losses of 5.2 ± 0.67 malate and 5.3 ± 0.41 calcium.

Both groups of experiments indicate that the utilization of calcium malate does not begin immediately. Inulin accumulates as the season advances, and disappears, along with any sugar that may be present initially, during starvation. The natural conclusion is that the carbohydrate reserves are drawn upon first, the calcium malate later. Injection of the tissues then soon supervenes, and progresses with the utilization of the malate.

Respiration of Stem

For comparison with the observations on malate content, the respiration of the stem was followed during starvation in continuous darkness. In some experiments the intake of oxygen, in others the output of carbon dioxide, was determined. The general features of the curves of respiration-rate were the same in all.

Intake of oxygen.

In an experiment (No. 53) at room temperature (15°–17° C.), the oxygen intake of a 1-year-old shoot in June fell from 1.94 (c.c. O₂ per hour per 100 gm.

fresh wt.), at first rapidly, then more slowly, to 0.5 by the 10th day, fluctuated between about 0.5 and 0.4 till the 25th day, then rose by the 30th day to 1.09. By this time the outer pith only had become injected. Fig. 2 represents the oxygen intake at 25° C. of a similar stem in January. After an initial fall

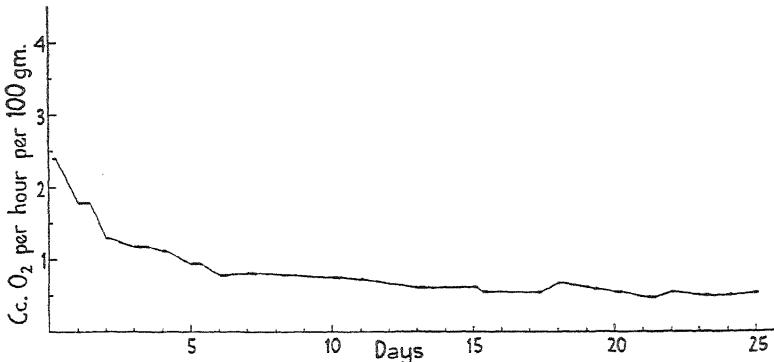


FIG. 2. Oxygen intake, 25° C. Expt. 50, Jan. 11, 1935; stem of 1933 autumn, 3.08 gm.

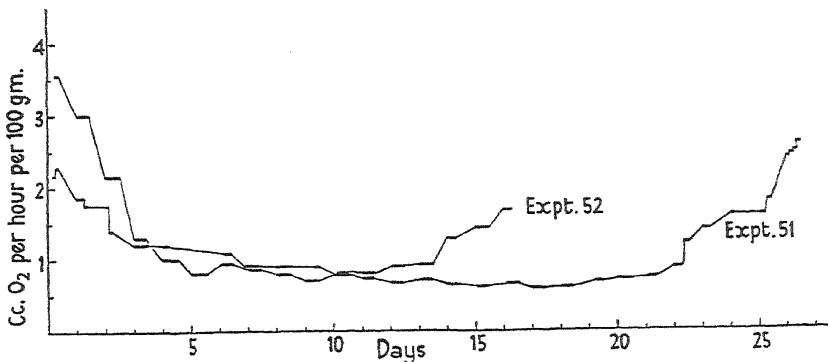


FIG. 3. Oxygen intake, 30° C. Expt. 51, Mar. 12, 1935; stem of 1933, 5.97 gm. After 18 days' appearance still normal; after 27 days, injected and moulds developing. Expt. 52, May 13, 1935; stem of 1934, 3.86 gm. After 16 days, pith injected and injection visible externally in patches; pH 8.0.

a low rate was still being maintained at the end of 25 days. Fig. 3 shows the oxygen intake at 30° C. of a 2-year-old stem in March (Expt. 51) and a stem of the previous autumn's growth in May (Expt. 52). The younger stem was respiring more vigorously, with less reserve: the rate began to rise after 12 days and by the 16th day injection was already visible externally. The later rise in Expt. 51, after 23 days, was also associated with injection; when examined after 28 days the stem was limp and moulds had begun to grow on the escaping sap. Two other short experiments (54 and 55) at 30° C. made

with the Dixon manometric apparatus in February and March, illustrated the initial fall in rate. Both initial rates were higher than in Expt. 52 at the same temperature for similar material in May; e.g. in Expt. 54 the rate began at 4·45 and fell to 0·95 within 5 days.

Output of CO₂.

As the apparatus for determining CO₂-output allowed the use of small amounts of material, the opportunity was taken of following the change of pH parallel with respiration. For Expt. 56 (Feb. 27, 1936) four large 2-year-old stems were cut into pieces 1 cm. long, the ends of which were waxed. Three of these pieces, weighing together 2·49 gm., were used for measuring the CO₂-output at room temperature (15°–16°C.); the remainder were left under similar conditions and the pH of samples determined by the B.D.H. capillarator method at intervals. The pH data so obtained were consistent and showed a very slow rise from 4·4–4·5 initially to 5·1 after 23 days. The pieces were by that time all used up except those in the respiration apparatus. The respiration was followed for another 11 days. By that time the rate was rising and the pH of the three pieces was found to be respectively 6·0, 7·7, and 8·5. This illustrates clearly the rapidity of the final rise in pH associated with injection.

Two other pairs of (simultaneous) experiments were made, one at room temperature, the other at 25°C. For each experiment a stem was divided into a larger middle portion, which was used for the determination of CO₂-output, the ends being waxed as usual, and smaller apical and basal portions of which the initial malate content was determined. The malate content of the middle portion was determined at the end of the period.

Fig. 4 gives one of the two closely concordant respiration curves for the first pair (Expt. 57, A and B: Apr. 22, 1936, 15°–17°C.). The stems were of the previous autumn's growth. The CO₂-output rate is of the same order as the O₂-intake rate in Expt. 53. The respiration was still continuing at a low level after 23 days, when the experiment terminated. Ten days later only the outer pith was injected, in B slightly more than in A. The malate data in mg. equivalents per 100 gm. fresh weight are as follows:

A: apex	7·5	base	14·6	average	11·05	middle	9·0	difference	2·05
B: "	9·7	"	15·7	"	12·7	"	9·1	"	3·6

Fig. 5 gives one of the two curves for Expt. 58 (similar material, in June; 25°C.). The initial rate of CO₂-output was much higher than the O₂-intake in Expt. 50 at the same temperature. The latter was, however, carried out in January: later results with another species indicate that such differences may be seasonal. Any wound reaction at the cut ends in Expt. 58 was probably slight.

The general form of the curves is similar, also the minimum level, about 0·5 after 25 days. At the end of 27 days the pieces had acquired a more

yellowish tint and the outer pith was injected. The malate data were as follows:

A: apex 7·2, base 13·2, average 10·2; middle 9·6; difference 0·6
 B: „ 8·4, „ 12·6, „ 10·5; „ 8·5; „ 2·0

In both pairs of experiments the small malate losses that are shown tally.

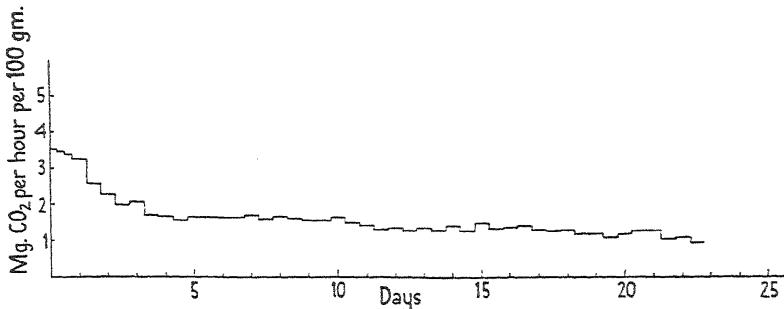


FIG. 4. Carbon dioxide output, 15-17° C. Expt. 57A, Apr. 22, 1936; stem of 1935, middle segment, waxed at both ends, 4·66 gm.

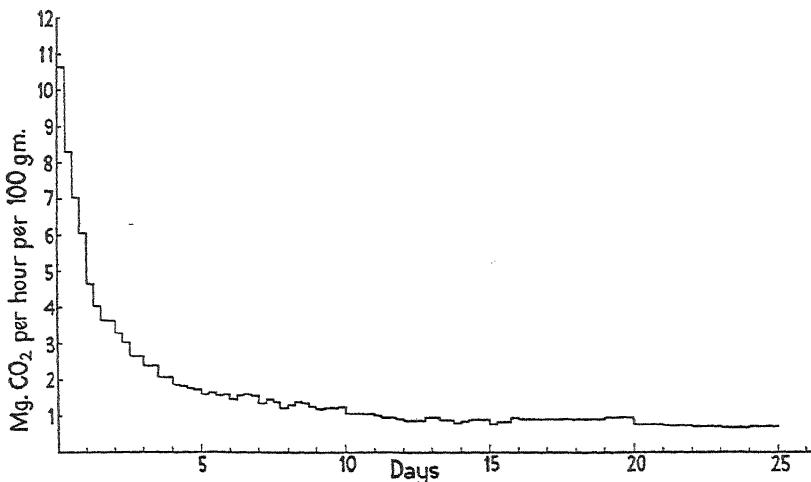


FIG. 5. Carbon dioxide output, 25° C. Expt. 58B, June 10, 1936; stem of 1935, middle segment, waxed at both ends, 7·18 gm.

with the early stage of injection, and the absence of any indication, except perhaps in Expt. 58A, of a final rise in the respiration rate.

Respiration of separated tissues.

As already remarked, the peculiar features of the pith, and the localization in it of the initiation of injection, made it appear desirable to examine its

respiration separately. Both oxygen intake and CO_2 -output have been followed, with excised pith and with outer tissues removed from the pith. Such experiments could not be prolonged beyond a few days as moulds developed sooner or later. For this reason, too, all the experiments were done at room temperature.

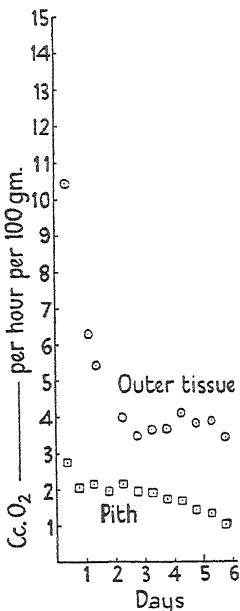


FIG. 6

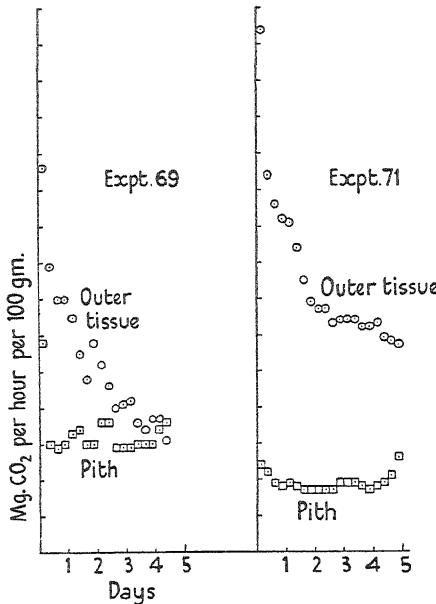


FIG. 7

Figs. 6 and 7. Fig. 6. Oxygen intake of separated tissues, 15° - 17° C. Expt. 65. Fig. 7. Carbon dioxide output of separated tissues, 15° - 17° C. Expts. 69 and 71. (As 1 c.c. CO_2 weighs about 1.85 mg. at 16° , the scale of ordinates should be reduced in this proportion for comparison with Fig. 6.)

The results range rather widely. For excised outer tissues the initial rate of oxygen intake was in some cases as high as 10 or 11 c.c. per hour per 100 gm. fresh weight, as compared with 1.94 for a whole stem in Expt. 53; initial CO_2 -outputs of 5.5 and 7.4 c.c. were found in Expts. 69-71 as compared with 3.8 in Expt. 56 and 1.8 in Expt. 57. The opening up of the tissues doubtless accounts for these high initial rates, partly through a purely physical diffusion effect, partly through a physiological reaction, to wound stimulus or readier exchange of gases. When cut surfaces are waxed no visible reaction follows in the stem of the plant. On the other hand, the difference between the initial rates in Expts. 56 and 57, already mentioned, may be the expression of a physiological reaction to wounding as such; but experiments directed specifically to this point have not yet been carried out.

In some experiments there are indications that the pith is not entirely

unaffected by excision. The initial rate of oxygen intake may be as high as 2.8 (Expt. 65) and fall gradually in five days to 1.2, or more rapidly to a lower level; sometimes it drops after the first day to 0.6 or 0.4. In other cases it maintains a low level from the beginning. In all cases, however, the respiration of the pith is far below that of the outer tissues. Fig. 6 (Expt. 65) is an example of oxygen intake; Fig. 7 gives the results of two experiments (69 and 71) on CO₂-output.

There were several instances in which no oxygen intake at all by excised pith samples could be detected. The possibility that pith may sometimes continue to serve as a store of sap after losing its protoplasm may therefore require examination. That pith is drawn upon for water is shown by the transverse fissuring which has already been recorded as occurring in old stems (Thoday and Woodhead, 1932).

DISCUSSION

The analyses recorded here have confirmed the presence of 'malic' acid in the outer cortex of the stem of *K. articulata* where no calcium malate is precipitated by alcohol, and have shown changes in total 'malate' between morning and evening corresponding to the diurnal changes in titratable acidity which characterize the metabolism of most succulent plants. These changes are, however, scarcely shown by the pith where calcium malate mainly accumulates. The diurnal periodicity may therefore characterize the non-calciferous tissues, rather than the stem as a whole. These are chiefly the green assimilating cortex, particularly the outer cortex, and the parenchyma of the bundle zone. In the pith the calcium malate is relatively immobile.

The starvation experiments present at first sight a rather confusing picture. This is attributable in part to seasonal changes, unavoidable in a succession of experiments each of long duration. The material used at any one time is also by no means uniform, notwithstanding special care in maintaining uniformity of conditions, and in the selection of cuttings.

Nevertheless, certain general results emerge from a scrutiny of the data. There is, first, the high correlation between the malate and calcium losses. This indicates that the malic acid associated with calcium is being removed and that the calcium is deposited in insoluble form.

Secondly there is the injection, primarily of the pith, by escaping sap, and, associated with it, a rapid rise of pH, which contrasts strongly with the small, very slow rise that occurs previously. It is a natural suggestion that these phenomena are consequential upon the destruction of malate, which, since that destroyed is associated with calcium, is mostly the malate located in the pith.

This might further be interpreted to mean that the utilization of malate marks a late stage in starvation, immediately prior to the final loss by the

protoplasm of its semipermeability. Reference to the titration curve of malic acid and to Bennet-Clark's diagram (1933, p. 39) of the relative proportions of different malate ions at different pH values shows that below pH 6 considerable changes in malate concentration could occur with small changes of pH owing to the buffering effect of the malate system itself. In the pith of *K. articulata* this appears to be the only effective system normally present. There is usually little or no phosphate to buffer the sap, so that a sharp end point is given with phenolphthalein. The outer tissues are by contrast strongly buffered, both phosphate and traces of aluminium being concerned in the effect (Thoday and Evans, 1931). Where, as may happen under conditions of abundant phosphate supply, phosphate is present in the pith as well as elsewhere, the rise of pH would be slowed down there, but normally the buffering is slight above pH 6.

How the calcium precipitation is to be fitted into the picture requires some consideration. According to Bennet-Clark's data malic acid is not completely neutralized until the pH reaches 8. Yet to explain the close correlation between malate losses and calcium losses calcium must be precipitated, as indeed the data for shorter periods of starvation indicate, before the pH of the sap as a whole rises conspicuously.

On the other hand, the processes go on in single cells, whereas the data available apply to tissues or whole stems. They therefore represent statistical averages, in which, for example, the rise of pH extending over a period depends in part upon the proportion of cells which may have reached a critical phase marked by high pH and escape of sap.

An indication of the relations to be expected can be obtained from experiments *in vitro*. From lime-water half neutralized by malic acid, carbon dioxide precipitates abundant calcium carbonate. When this precipitation is complete, further treatment with CO₂, by breathing through the solution, rapidly lowers the pH to about 6.2. Boiling, after decanting from the precipitate, to drive off excess of CO₂, raises the pH to about 8.5. This is the pH of distilled water saturated with CaCO₃.

Neutral malates of calcium have a limited solubility in water, though the naturally occurring *l*-malate is more soluble than the inactive *dl*-malate. Acid malates are readily soluble. Removal of malate will thus have as one of its consequences, along with a rise in pH, a lowering of solubility of the calcium malate. It is of interest that the solubility of the *l*-malate is of the order of 10 mg. equivs. per 100 c.c. (at 20° C.; a little less at 30° and at 15°).¹ The concentration of calcium found is often higher than this, up to 15 mg. equivs. or more per 100 c.c. In individual cells still higher concentrations must occur, in view of the unequal distribution revealed by precipitation *in situ*. Crystallization of neutral calcium malate could therefore occur.

This can be illustrated at a lower level of solubility with *dl*-malate, which dissolves to the extent of 3 mg. equivs. per 100 c.c. at 15° C.¹ If to 1 per cent.

¹ Solubility data from International Critical Tables, 1927, vol. iv.

malic acid (about 14·9 mg. per 100 c.c.) CaCO_3 is added, Ca malate crystallizes out in abundance while the reaction is still acid.

Such crystallization, however, is not likely to affect the results obtained here, by analysis of water extracts, as any crystallized *L*-malate would be redissolved during extraction.

Precipitation in insoluble form must depend on the formation of CaCO_3 . In equilibrium with atmospheric air the solubility of CaCO_3 is only 0·13 mg. equivs. per 100 c.c. Higher concentrations of CO_2 in the sap would keep more calcium in solution. (In equilibrium with 1 atm. CO_2 the solubility is 2·3 mg. equivs. per 100 c.c.) The pH might also be kept down in the neighbourhood of 6. But a solution of neutral calcium malate in contact with calcium carbonate, left exposed to the air, loses CO_2 and assumes a pH between 8·5 and 9. This is the pH of injected parts of the pith.

Given free diffusion of CO_2 , a pH of 8·5 should correspond to the beginning of CaCO_3 precipitation. This point should soon be reached once decomposition of malate begins. Reference to titration data (Thoday and Evans, 1931) shows that a very small addition of alkali suffices to raise the pH of a malate solution above 6. For a 0·2 per cent. solution (= 3 mg. equivs. per 100 c.c.) only 0·12 mg. equiv. per 100 c.c. was required to change the pH from 5·5 to 8·5 (loc. cit., p. 66). A loss of malate to the extent of only 4 per cent. of the total malate present would effect the same pH change.

On the other hand, the observations suggest strongly that injection and high pH are associated with depletion. It is clear from the low final concentrations of calcium and malate obtained in some cases and indicated by micro-chemical observations on injected pith that utilization of malate can proceed far beyond the point assumed in the previous paragraph. It is difficult to avoid the conclusion that utilization of malate proceeds continuously and that the pH is kept from rising by the accumulation of CO_2 —the diffusion of CO_2 *not* being free. The rise of pH would then be associated with the exhaustion of the cell, the cessation of respiration and the escape of the excess of CO_2 which is no longer renewed. This, however, leaves to be explained the final rise in respiration rate shown in all the experiments which were continued long enough.

The respiration experiments with separated tissues have shown that the pith respires at a much lower level than the remaining tissues. The respiration curves for the stem as a whole thus correspond, at any rate in their major features, to that of the outer tissues; the respiration of the pith, although it forms about half the bulk of the stem, will affect the form of the curves to a smaller extent.

The initial phase of rapidly falling rate in particular is to be referred to the outer tissues, excised pith having shown it less markedly if at all. It corresponds to the similar phase described by F. F. Blackman (1908, p. 891) for leaves of Cherry Laurel and *Tropaeolum*. In these leaves it is followed, after a period of minimal respiration, by a rise to a peak and a subsequent

fall, during which the colour of the leaves changes from green to yellow. Yemm (1935) has found a similar behaviour in barley leaves.

Godwin and Bishop (1927) state with regard to Cherry Laurel that the presence and extent of this respiration 'hump' varies with the age of the leaves. Young leaves show no hump and do not turn yellow; when the respiration rises they begin to turn brown, as do leaves of all ages at the onset of the final phase of starvation. With increasing age this final phase is progressively delayed, and yellowing, with the associated hump increasing in prominence, is interpolated.

Yellowing is not a feature of the starvation of *Kleinia articulata* stems. A slight change of colour towards yellowish green has been recorded in one experiment, but in this case no rise in respiration had appeared. Generally, injection with sap occurs while the outer tissues are still green. Outward spread of sap escaping from the pith cells would complicate the phenomena and vitiate close comparison with starving leaves. The late rise in respiration is, moreover, not to be compared closely with the final rise in respiration shown by Cherry Laurel leaves, for in these the sap escapes to the surface and the rise is attributed to moulds. In *K. articulata* stems the injection is at first wholly internal and mould development is only observed at an advanced stage of disintegration, when injection is general and sap escapes to the surface. For the same reason, as well as in view of the maturity of the stems, it does not seem profitable to compare their behaviour with that of young leaves. It is indeed possible, either that the rise in respiration is connected with breakdown processes in the pith, or that it represents the effect of injection on the respiration of the outer tissues, bringing the starvation process there to an untimely end. The latter alternative fits in with the conclusion suggested by consideration of malate and pH relations and seems therefore the more probable.

A feature of the respiration curves which is somewhat surprising in view of the normal diurnal cycle of acidity is the absence of initial fluctuations in respiration such as were observed by Bennet-Clark (1932) for the succulent leaves of *Sedum praealtum* and *Crassula lactea*. The determination of oxygen intake was interrupted at night and the oxygen intake curves are therefore not conclusive; but the carbon dioxide output curves represent continuous series of determinations (see, e.g., Expt. 58, Fig. 6).

Estimation of the significance of such features is made difficult by the lack of uniformity in the material, to which reference was made at the outset. Different parts of the same stem differ in age, past history, amount of reserves, and other accumulations. The different tissues and even parts of the same tissue in one segment of the stem also differ. Even if phases in the respiratory metabolism of the cells, say, of the pith were sharply marked, the fact that the onset of a phase did not coincide in all the cells would tend to obscure it. The long duration of experiments with relatively sluggish stem material, and the difficulty of seasonal variations, were additional considerations,

which made it appear more profitable at this stage to defer any attempt to analyse the phenomena further in the stem and turn to leaves.

Complete uniformity and coincidence of phases are of course not to be expected. Even in ordinary leaves, consisting predominantly of mesophyll, the respiration hump is partly a statistical result of the consecutive incidence of changes associated with yellowing of cells in different parts of the leaf. The peak occurs when the largest number of cells are undergoing the change at the same time. The rise in respiration of individual cells must be of much shorter duration.

Nevertheless, succulent leaves are far more homogeneous than stems. All the tissues are much more nearly of the same age, and it may be anticipated that the slurring of the evidence of change by the spread of its incidence in time will be far less.

It has been found (Jackson and Thoday, 1939) that the leaves of some species of *Kleinia* resemble the stem of *K. articulata* in the accumulation of calcium malate in a parenchymatous water tissue, which is surrounded by the peripheral, calcium-free clorenchyma. Leaves of *K. radicans* were chosen for subsequent experiments, an account of which will be given in the second part.

SUMMARY

Determinations of total malate at different seasons and times of day in leaf and stem of *Kleinia articulata* and in different tissues accord with previous determinations of titratable acidity and microchemical observations.

Reduction of malate content during starvation in the dark was demonstrated. Loss of sap calcium was also found, highly correlated with the malate loss. At a late stage of starvation, when the pith and (later) other tissues become injected with sap, the pH rises sharply to between 8 and 9. This may signify cessation of respiration in the pith, respiratory CO₂ having previously maintained a pH not above 6. Calcium is probably precipitated as carbonate.

Stem respiration falls rapidly during the first few days to a low level which is maintained fairly steadily until injection is advancing, when a rapid rise occurs. The starvation curves differ from those of leaves by the absence of any interpolated rise in respiration; no yellowing of the green tissue occurs. It is suggested that the final rise may represent the effect of injection by pith sap on the outer tissues, bringing the course of their starvation to a premature end.

Notwithstanding the normal diurnal periodicity in acid content, no fluctuations in respiration rate were observed such as Bennet-Clark found during the first few days of starvation with leaves of Crassulaceae. Excised pith respires at a low rate. The outer tissues alone showed the initial rapid fall which characterizes the respiration curve of the intact stem.

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A Bifurcated Inflorescence of *Digitalis purpurea* L.

BY

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With eight Figures in the Text

AMONG the numerous records of abnormal development in the genus *Digitalis* I have been able to trace only two references to forking of the inflorescence axis. Both of these are contained in one paper (Godron, 1871) and relate to hybrids of which one parent was *Digitalis lutea*. One hybrid arose spontaneously, the second parent being unknown. The other was the outcome of an artificial cross, *D. lutea* × *D. grandiflora*. Both plants had an inflorescence axis which forked into two branches, the axes above as well as below the forks being fasciated. In relation to the former plant Godron wrote that the fasciation commences below the inflorescence and attains its greatest breadth (50 mm.) towards the middle of the cluster. It is forked at the top and the branches of the fork are fasciated and of equal length. The unilateral flowers are on the same plane as the fasciation.

Numerous other instances of forking associated with fasciation are to be found in the literature (Godron, 1871 and 1874; Johnson, 1926; Worsdell, 1905), and Worsdell (1905) makes the statement that 'the strap shaped shoot always subdivides at the apex into two or more shoots of equal value'. On that account, according to Knox (1908), 'fasciation has been considered as including two tendencies, one to enlargement and the other to division'. In a paper which appeared while the present one was in course of preparation Bausor (1937) gives the impression that he regards division as antithetic to fasciation, for he describes three ways in which fasciated shoots of *Phaseolus* may become normal by division: by fissure; by forking of the apical meristem; by leaf sectoring. That division does not necessarily lead to normal stems is evident from records of Godron and others of fasciated stems which fork to produce fasciated branches.

The plant which is the subject of this paper was self-sown in 1936 on the top of a rockery. It appeared among a number of others on a site occupied during the previous season by a wild plant brought in from the field. It conformed in all but the abnormality to be described with the species *Digitalis purpurea* L.

[*Annals of Botany, N.S. Vol. III, No. 11, July 1939.*]

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No plants of *Digitalis lutea* could be found anywhere in the neighbourhood, so there is no reason to suppose the abnormal plant had that species in its immediate ancestry. *D. lutea* is not a British plant, and it is unlikely that the wild stock included any hybrid forms. Hence one is led to the belief that

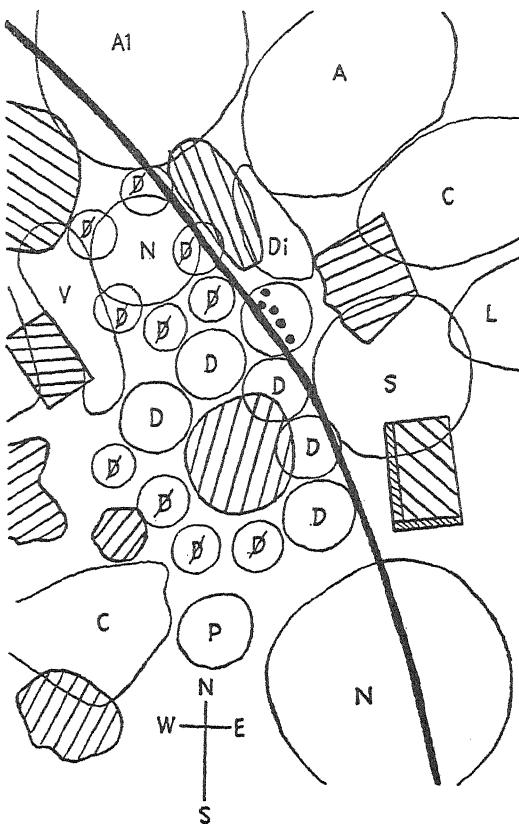


FIG. 1. Position of bifurcated plant. Hatched areas represent 'rock'. Heavy black line marks the crest of the bed. Circle with five dots shows position of forked plant; dots correspond to the apices of the stems. Other plants marked: A1, *Alyssum*; A, *Arabis*; C, *Cistus*; D, *Digitalis*; Di, *Dianthus*, removed 1936; L, *Lithospermum*; N, *Nepeta*; P, *Phlox*; S, *Sedum*; V, *Viola*.

bifurcation of the axis of *Digitalis* is not necessarily correlated with *D. lutea* parentage.

The situation in which the plant grew is indicated in the sketch-plan of the surrounding part of the rockery (Fig. 1). This is a low ridge running approximately north-west and south-east, with a slope of 1 in $2\frac{1}{2}$ to the north-east and 1 in $3\frac{1}{2}$ -4 to the south-west. The soil is mainly rotted turf and meadow loam and the 'rock' material around the abnormal plant chiefly broken concrete. The bifurcated plant was rooted just below the ridge of the rockery

on the north-east side. Below it to the north-east there is a short steep drop to a flat step, so the plant would tend to receive rather less water than its normal fellows which grew on the more gradual south-west slope.

In 1936, when the plant was in the seedling stage, it was closely surrounded on the south-east, east, north-east, north, and north-west by plants of *Sedum spectabile*, *Cistus*, *Dianthus*, *Arabis*, and *Alyssum*, and on the remaining sides by seedlings of its own kind. During the autumn clearance, much of the growth on the east, north-east, and north was cut down and many of the foxglove seedlings on the north-west were removed. The plant was thus exposed to the colder winter winds, while it was still shaded by the plants to the south and west.

In 1936 nothing unusual was noticed among the seedlings and a photograph, taken in that year, shows that the seedlings were well grown though closely crowded. In June 1937 the plant bore a main inflorescence axis with four distinct forks (Fig. 2). One occurred among the basal vegetative leaves, two others arose in the flower-bearing regions of the branches formed by this first fork, and a fourth appeared a few centimetres above one of the secondary forks. In July the tallest branch (Fig. 2, B2b) began to fork again. The pair of buds found there, together with a short length of the axis on whose apex they arose, was removed, preserved, and later microtomed. Both buds had axes flattened in their upper parts as if tending towards yet further bifurcation.

It may be noted here that the plane of forking was roughly parallel with the crest of the bed in which the plant grew (Fig. 1).

In addition to the forked central axis the plant produced three lateral axes from low down in the vegetative region. They were all perfectly normal in appearance. Two are to be seen clearly in Fig. 2 standing out to the right of the plant. The third was bent down at the time of photographing under the *Sedum* to the left so as to clear the view of the central inflorescence.

This tendency to stronger development on the 'right-hand side' seen in the lateral branches is also evident in the forked central inflorescence. That side of the whole plant is the taller, and of any two branches produced by a bifurcation the right-hand one branches again more than the left.

MORPHOLOGY OF THE AXIS

All parts of the forked axis appeared somewhat flattened and provided with superficial ridges, though these were more pronounced in the lower parts than in the upper. Dimensions of the parts as determined by means of rule and callipers are set out in Table I. The measurements were made after the stem had been cut and had dried in the air while the phyllotaxis was being investigated. The dimensions are therefore smaller than they would have been if taken from fresh material, but as shrinkage seems to have been uniform, they give the proportions satisfactorily.

The measurements confirm the visual impression that the axes are flattened. They also indicate that forking results in branches of approximately the same

girth. On the other hand, if we multiply the least thickness by the greatest thickness, and so get the measures of the sectional areas given in the last

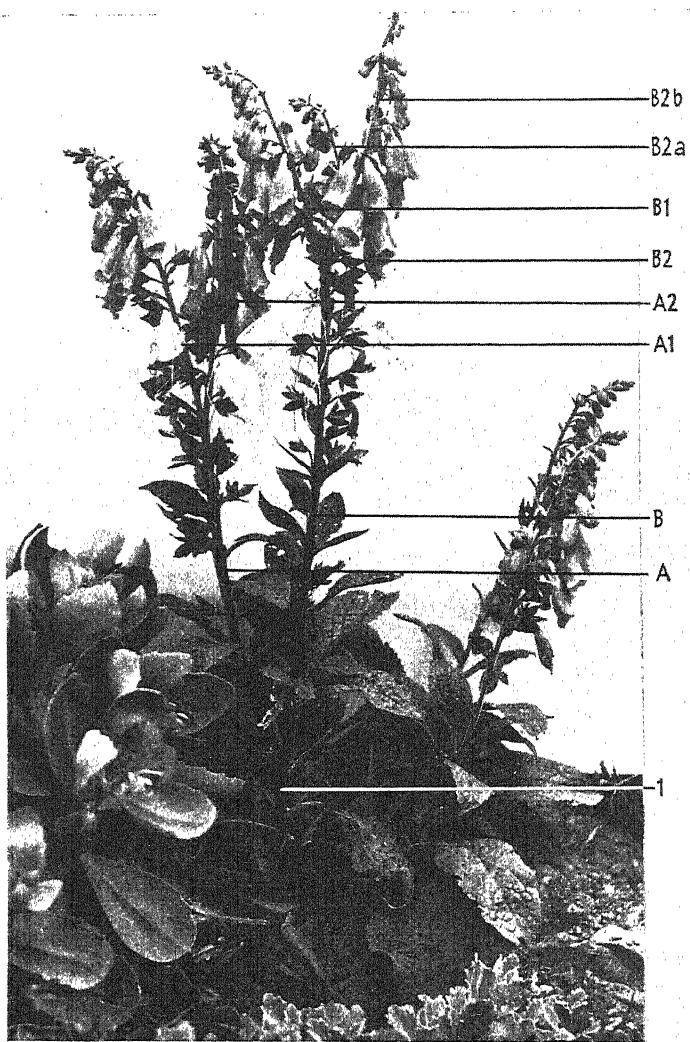


FIG. 2. A bifurcated inflorescence of *Digitalis purpurea*. The figures and letters marking the branches are used in the text to facilitate reference.

column of the table, it becomes apparent that of two branches of the same order, that which branches again to the greatest extent higher up has the larger sectional area at its origin. It is the right-hand branch of each pair which has the larger area.

TABLE I

Axis Dimensions

Axis.	Length, cm.	Least thickness		Greatest thickness		Product.
		Bottom, cm.	Top, cm.	Bottom, cm.	Top, cm.	
I	8.5	—	0.5	—	0.9	0.45
A	18.5	—	0.35	—	0.46	0.161
B	31.5	0.46	—	0.57	—	0.2622
B ₁	—	0.46	0.30	—	0.50	0.15
A ₁	26.0	0.22	—	0.36	—	0.276
A ₂	27.0	0.19	—	0.35	—	0.0792
B ₁	19.0	0.24	—	0.28	—	0.0665
B ₂	4.5	—	0.23	—	0.38	0.0672
B _{2a}	9.5	0.24	—	0.32	—	0.0874
B _{2b}	11.5	0.15	—	0.17	—	0.0768
		0.19	—	0.26	—	0.0255
						0.0494

In the axis column, I signifies the axis up to the first fork; A and B, the branches formed by this fork, A being the one to the left in Fig. 2; A₁ and A₂ are similarly branches of A; B₁ and B₂ of B; B_{2a} and B_{2b} of B₂. Dimensions of the tops of axes which terminate in buds are not given.

TABLE II

Flatness of Axis Segments

Axis segment.	Number of subsequent forks.	Flatness of segment		Increase in flatness.
		Bottom.	Top.	
I	5	—	1.8	—
A	1	1.26	1.31	0.05
B	3	1.30	1.66	0.36
A ₁	0	1.66	—	—
A ₂	0	1.84	—	—
B ₁	0	1.17	—	—
B ₂	2	1.33	1.65	0.32
B _{2a}	0	1.13	—	—
B _{2b}	1	1.37	—	—

Expressions of the degree of flatness of the various segments obtained by dividing greatest thickness by least thickness are given in Table II. They show that at all forks but one there is a reduction but not an elimination of flatness; the flatness of the top of segment I is 1.8 and of the bottoms of segments A and B 1.26 and 1.30 respectively, and so on. That this is not true of the one exceptional fork, A-A₁, A₂, is probably because of special conditions there; the segments A₁ and A₂ seem to have been delayed in separating and, as Fig. 2 shows, were twisted through 180 degrees as they separated. The figures for flatness also indicate that in each instance where top and bottom of a segment could be measured flatness increases from bottom to top of the segment. Moreover, there is a rough parallel between number of

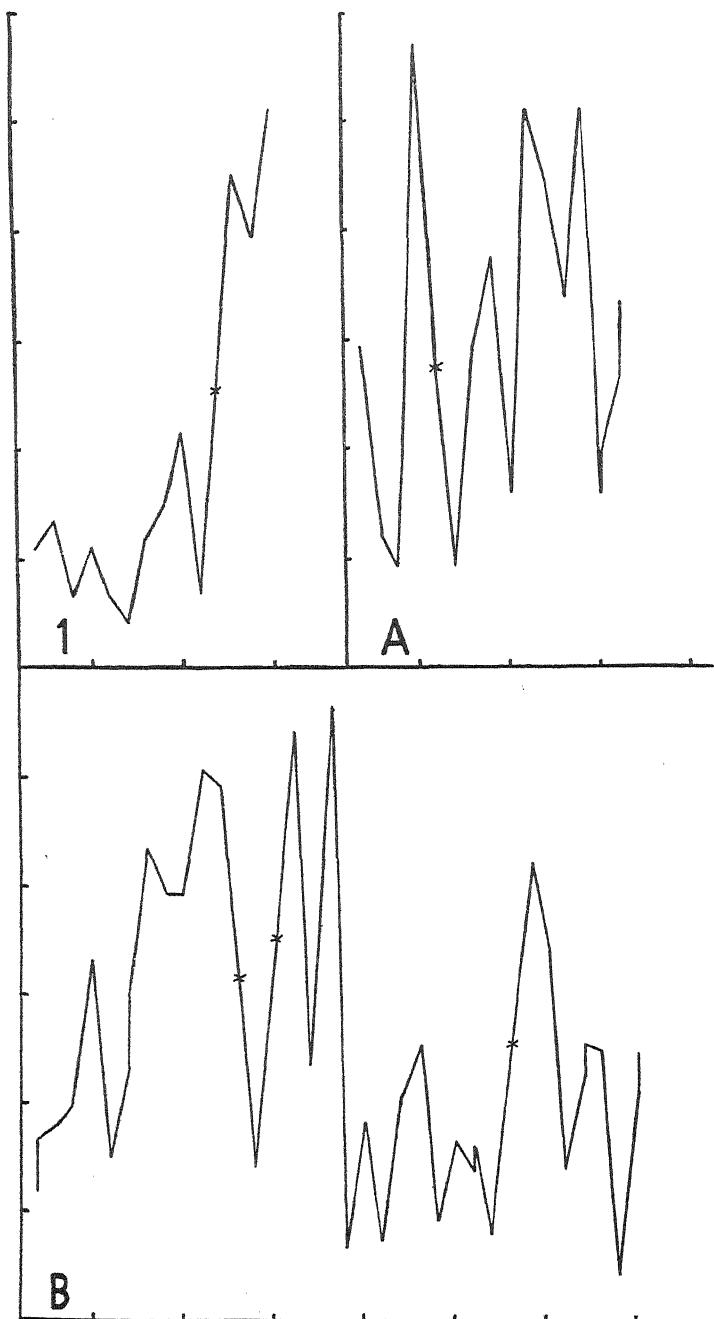


FIG. 3. Graphs of phyllotaxis of stem segments 1, A, and B. For explanation see text.

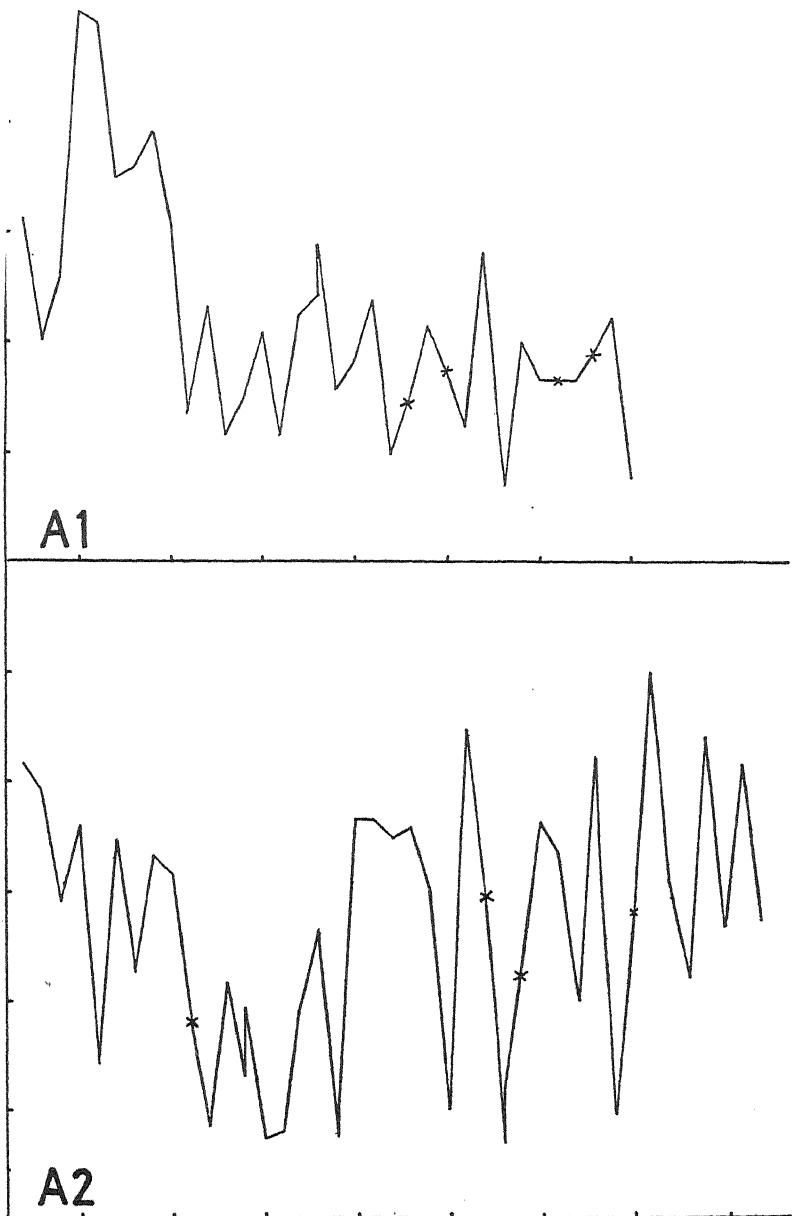


FIG. 4. Graphs of phyllotaxis of stem segments A1 and A2. For explanation see text.

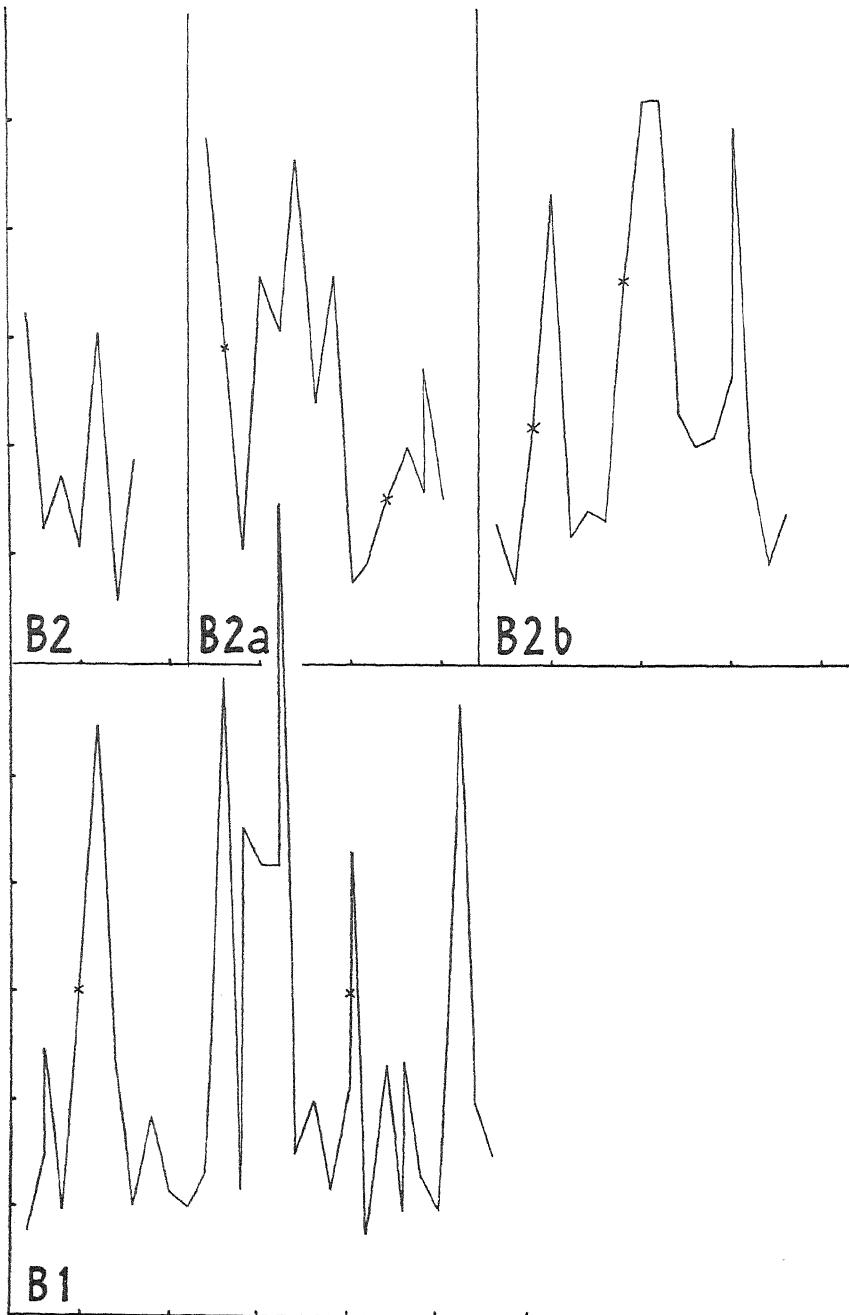


FIG. 5. Graphs of phyllotaxis of stem segments B2, B2a, B2b, and B1. For explanation see text.

later branches and flatness of segment top (A, B₂, B, 1) as well as of increase of flatness in segment length.

From ground-level to the first fork the ridges on the surface of the stem follow closely the general direction of growth; they are parallel with the sides of the stem. Above the first fork, especially in the middle region of the inflorescence, biastrepy is obvious; the ridges there make long spiral turns in an anti-clockwise direction around the stem, suggesting torsion.

PHYLLOTAXIS

The leaf attachment was very variable and irregular. Vegetative leaves on the lower part of the axis and bracts on the upper part arose from all sides of the stems, but the spiral connecting the leaves in places made long turns on one side of the stem and alternating short ones on the other, while leaves in pairs at the same height were not uncommon and in one or two instances fusions between leaf-bases inserted at different levels had occurred.

An attempt was made to elucidate any laws which might control the phyllotaxis by plotting the positions of the leaves on to spirals and subsequently setting off in a graph the angles between consecutive leaves, so determined, against numbers indicating the sequence of the leaves on the stem. Figs. 3, 4, and 5 reproduce graphs so obtained. Along the abscissae each marked division corresponds to five leaves, and up the ordinates each marked division represents 60 degrees.

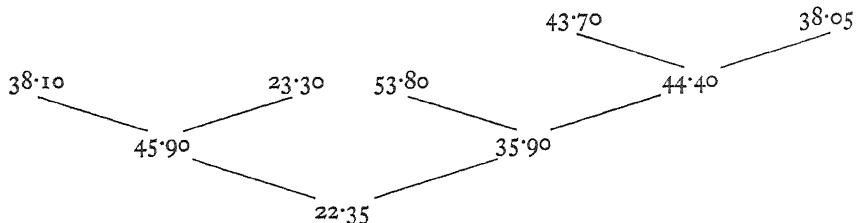
Were the phyllotaxis a regular spiral the graph would be a straight, horizontal line, and hence the extreme irregularity of the curves illustrates the irregularity of the phyllotaxis. It does not seem probable that any simple law could be framed to express such irregularity.

Even an attempt to express the quantity of the irregularity meets with difficulties, the chief of which is lack of a standard. It is not possible to study deviation from normal in the plant itself since it was completely abnormal when first observed. One cannot use as a standard the normal phyllotaxis of the species: it does not exist. Examination of numerous wild specimens showed spirals of 1/3, 2/3, 2/5, 3/8, and 4/7. Neither is it permissible to use as a standard the phyllotaxis of the apparently normal lateral branches, since in the wild plant this usually differs from that of the central inflorescence.

It does, however, seem possible to obtain a comparative measure of irregularity from the phyllotaxis graphs. If we plot in the manner described above any regular spiral phyllotaxis, no matter at what angle the successive leaves are set, the result will always be a horizontal line. Dividing the length of the line by the number of leaves plotted will always give the scale of the ordinates of the graph. Clearly, if one leaf is displaced from its wonted position in relation to a preceding one, the graph line joining the two leaves will slope instead of being horizontal and hence be longer. Thus if we measure the total length of a graph of irregular phyllotaxis and divide by the number of leaves plotted, we shall obtain a figure for the average deviation from some

unknown normal position which may be compared with other figures similarly obtained and with the scale of the abscissae which represents regularity. It may seem that the occurrence of two or more leaves having equal or similar displacement would vitiate the method, and that the greater the number of leaves equally or similarly displaced that occur the less accurately would the method describe the irregularity. Since, however, there is no normal phyllotaxis, in the extreme case when many successive leaves are similarly displaced one would be forced to consider them as occupying new normal positions. In a like manner, when a few successive leaves are similarly displaced, one should consider that a change in phyllotaxis has occurred. Irregularity occurs and is measured in the changing region and not in the modified one.

The diagram below sets out according to their position on the plant the average irregularities of the various stem segments. It is evident that the irregularity is considerable; the smallest figure, 22.35, would also be given were similar calculations made from an axis bearing the same number of leaves which were displaced about 25 degrees alternately on either side of their normal position.



Irregularity of phyllotaxis according to position on plant.

The one-sided development of the plant is again apparent: in each instance the right-hand fork has a smaller irregularity. It may also be noted that the irregularity waxes and then wanes as we progress up the inflorescence.

Only one feature of leaf attachment appeared constant: the first leaf above each bifurcation arose in the crutch of the fork. In all instances except one the leaf-base straddled the fork a little to one side of its midline in such a way as to give the impression that it sprang from the base of one of the branches at the junction of this with its fellow. The one exception, fork B-B₁, B₂, showed a leaf arising a very short distance up one branch, B₁ (Fig. 6).

BUD SECTIONS

Serial sections 10 μ thick were prepared from the pair of buds removed from the apex of the stem segment B_{2b}.

In the lowermost sections, obtained from close to the level of severance of the material from the top of B_{2b}, the axis is seen to be flattened (Fig. 7, L). In higher sections it becomes even more so till just below the bifurcation it attains a flatness of 6.6. Each of the branches produced by the forking is

flattened. It is thus evident that in the early stages of the development as in the mature condition bifurcation reduces but does not eliminate fasciation.

The phyllotaxis was found to be just as irregular in the buds as in the mature stems, and in the crutch of the bifurcation of B_{2b} which gave rise

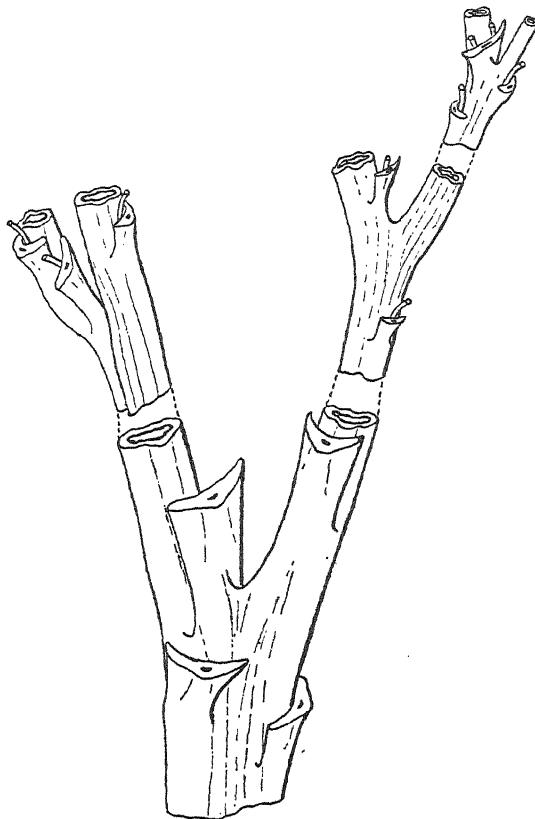


FIG. 6. The crutches of the bifurcated axis showing leaf attachment.

to the two buds a leaf is attached. Its attachment resembled that already described for leaves occupying similar positions in older parts of the plant. The vascular supply in connexion with this leaf arose in part from one side of the flattened stele of B_{2b} and in part from the opposite side. With its emergence the stele becomes divided into two whose upper ends terminate in the two buds.

In *Digitalis* the procambium becomes connected into a ring very early on, but in the youngest parts of buds isolated procambial strands may be seen. In them the protoxylem groups differentiate and these are readily distinguishable for some time. One of them enters the base of each bract leaf and

subsequently divides. Two others, one from each side of the leaf trace, pass out at the node to enter the flower-stalk and there coalesce (Fig. 7, G-C).

Examination of the sections in the region of the B₂b-bud crutch shows that three protoxylem groups associated with the crutch leaf can be distinguished

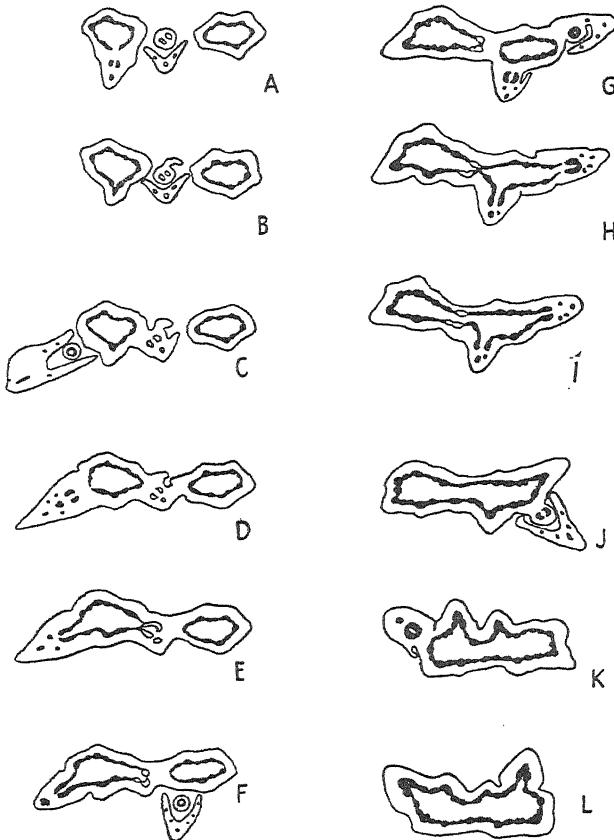


FIG. 7. Sections of the B₂b-bud crutch. Selected sections drawn under the camera lucida. Leaf sections are depicted only when necessary to explain changes in the stem. Crutch leaf bundles are drawn in outline, other bundles are blocked in.

easily some 0·46 mm. below the crutch surface. At that level (Fig. 7, I) two, one large one which ultimately passes out into the leaf, and one small one destined for the flower-stalk, appear midway along one of the broader sides of the flattened stem. The third, which will complete the bud supply, is situate opposite its fellow on the opposite side of the stele. At lower levels in B₂b these three bundles merge with others arising on the same sides of the stem as they occupy respectively in section I. At a higher level the procambium joining the leaf-bundle to its neighbours on one side of the stem

and that in contact with the solitary bundle on the other approach one another. Higher still they meet (Fig. 7, H). The solitary bundle is thus brought into propinquity with its leaf and fellow bud bundles, and when this occurs the stele becomes divided into two. Two somewhat flattened steles are seen above this level with the three bundles related to the crutch leaf on the inner edge of one of them (Fig. 7, G). The part of the stele containing the three bundles becomes twisted towards the side of the stem in which two of them were originally noticed, and in due course separates off to form the traces of the crutch leaf and its axillary bud (Fig. 7, F-A).

In the highest section obtained of one of the buds a state of affairs similar to that which has just been described was found in the course of one of the procambial strands. The stem apex shown in the uppermost section in Fig. 8 contains one procambial strand. In the next section two other strands are visible in contact with this, one on either side. These are the bud procambia. The three constitute the vascular supply, related to what is believed would have become a crutch leaf had the bud been allowed to continue to grow. Their counterpart, related to the leaf whose tip is shown above the uppermost section, may be seen in the three procambial strands depicted on the opposite side of the second section. As the three 'crutch leaf' strands pass into the third section the leaf trace and the 'left-hand' bud strand move to a position deeper within the stem. The bud trace continues this movement across the stem in the fourth, fifth, and sixth sections, until in this last it is seen to have crossed the developing stele and united with the bud strand associated with the leaf forming on the opposite side of the stem.

It is of interest to notice that development in the stem apex is further advanced to the 'right' than it is to the 'left' of the crossing procambial strand. Section 5 shows at its 'right-hand' extremity the base of a fully formed leaf and just behind the crutch leaf strands the base of another approaching that state, while in corresponding positions to the left of the section protuberances only of the surface mark where two leaves would in due course have arisen. The procambial strands associated with these are to be seen in the sixth section, and that is the highest level at which procambium appears on the 'left'. The very apex of the meristem is higher on the 'right' than on the 'left'.

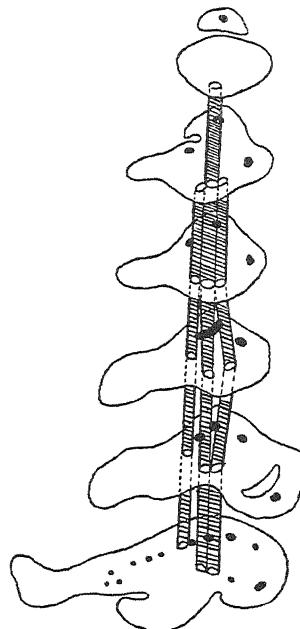


FIG. 8. The uppermost six sections of one of the buds.

DISCUSSION

Nature of fasciation.

The original meaning of the term fasciation was that of a bundle of coherent shoots. Hinks (1853) believed that fasciation arose by the adhesion of a number of buds, Masters (1869) that it resulted from the adnation of a number of shoots, while Linnaeus (1751) held that the formation of an abnormal number of growing-points which became coherent led to the condition. As Worsdell (1905) points out, there was underlying these beliefs the idea of the union of structures already delimited. He favours a view involving the failure of separation of organs which in the normal condition are distinct, for, he says, 'the fasciated organ represents from birth onwards the equivalent of two or more organs of which at the earliest stages there was absolutely no sign. These latent organs assert themselves gradually.' Conceptions directly opposed to those of fusion, whether postgenital or congenital, were held by Moquin-Tandon (1841) and Nestler (1894), who believed that the condition was due to the activity of a single growing-point. Nestler, in fact, states that he could find no evidence of separate growing-points or of congenital adhesion. It was Moquin-Tandon's view that the enlargement of a single growing-point led to fasciation: Knox (1908) found that fasciations in *Oenothera biennis* arose after the partial destruction of the stem apex, and Compton (1911) believed that sterilization of part of the apical meristem of the Mummy Pea produced the condition.

Bifurcation, which is so common a correlation of fasciation, would result from the ultimate separation of buds, shoots, or growing-points of Hinks, Masters, and Linnaeus or from the ultimate expression of the individuality of the latent organs of Worsdell. On the other hand, according to the contrasting view of Moquin-Tandon and Nestler no such organs exist; bifurcation on this basis must be initiated by the division of one entity.

According to the fusion theories the forked inflorescence under consideration would have arisen in one or two ways. Numerous branches would be considered to have fused during the rosette stage of the plant to separate at intervals into paired groups in the inflorescence axis: alternatively, it might be supposed that lateral branches had grown adnate to the parent stem for some distance before separating.

If the former suggestion were true, the primary apex of the plant must have aborted, and there is no evidence of this, or else have united with at least five laterals to provide, in all, the six apices of the inflorescence. Whether abortion of the apex or its union with laterals occurred to form a plant such as has been described, the primary bunch of stems must have divided five times into *two* bunches, never more; and one can see no reason why, when the bunch was breaking up, it should not at least sometimes separate into three or more parts. Moreover, each time a bifurcation occurred, the right-hand fork would contain more stems than the left despite the observed fact

that the diameters of the forks are approximately the same. Again, it seems reasonable to suppose that the bigger the number of stems in any branch the greater would be the irregularity of the phyllotaxis. This is the opposite of the observed fact that the right-hand branches of the forks which branch again more than the left have smaller irregularities (see diagram, p. 708).

The second suggestion, that of fusion of laterals, seems just as improbable. If it had occurred one would expect to find lengths of the axis with regular phyllotaxis terminating where the lateral arose and being succeeded by lengths with lesser regularity till separation of the lateral adnate branch restored the regularity. No such condition exists in the whole plant.

There thus remains only one alternative: the fasciated stem must have arisen from one growing-point which suffered splitting at intervals. The direct evidence for this belief rests in the bud sections. Those of the B₂b-bud crutch (Fig. 7) illustrate the bifurcation of a flattened stem which it is reasonable to suppose arose from an apex similar to that illustrated in Fig. 8. Here we are undoubtedly concerned with one flattened meristem. Similar conditions were found in the second bud of the pair, and there are to be seen in neither of these buds any evidence of fusion of organs.

The cause of fasciation.

The earliest explanations of the phenomenon were obviously based on the appearance of the fasciated shoot. Thus Godron (1872) speaks of it as due to 'excess of life manifesting itself at the edges and top of the organ'. Sachs (1892), de Vries (1889), and Goebel (1900) state that when the main axes of germinating seedlings are injured the lateral shoots frequently become flattened. The operation would seem to result in a sudden increase of food-supplies to the lateral buds. Similar experiments performed by Reed (1912) produced fasciations in seedlings with hypogaeal germination, but not when those with epigeal germination were used. He deduced some difference in the availability of the food reserves on this account. One can see underlying these explanations the recognition of the extra amount of food necessary for the growth of the enlarged organs, but no clear proof that food-supply was the primary cause.

Worsdell (1905) suggested that fasciated plants must have some individual idiosyncrasy since neighbouring plants of the same kind though growing under the same conditions are not all fasciated. Indeed, fasciation is known to be heritable in *Celosia* (Knight, 1822) and in *Cirsium* (Moquin-Tandon, 1841). In *Crepis biennis* de Vries (1889) found a portion only of the offspring of fasciated plants to be fasciated.

Hus (1908) writing in reference to fasciation says that mere excess of nutrition is not sufficient to explain the inheritance of the character, and Godron (1872) that fasciation is seldom heritable and is never so in an absolute manner. Knox (1908) found in *Oenothera* the incidence of fasciation no greater in the offspring of fasciated than in the offspring of non-fasciated.

She ascribes the condition to disturbance of physiological balance in the growing cells due to insect action which results in the multiplication of cells, stems, and leaves. Other workers such as Peyritsch (1888), Petch (1911), and Johnson (1926) find a similar primary stimulus in the action of insects, fungi, or X-rays.

The cause of fasciation in the present instance must remain uncertain. The facts do not fit closely with any of the previous explanations. The situation of the plant would lead one to the belief that it was not more favourably placed for nutrition, probably less so, than its non-fasciated neighbours; hence it does not seem probable that excessive nourishment was the cause. There is no evidence of destruction of an apex which might lead to diversion of supplies, though there might have been an augmentation of such supply when on account of the autumn clearance the plant was given access to more light, &c. It is true that neighbouring plants were subject to the same change and yet remained normal, but these growing as they did on the more generally favourable south-west slope would perhaps not be subject to such an intense change as was the fasciated plant on the north-east slope.

The possibility that the forked plant had a heritable tendency to fasciate which would allow the same change in conditions to affect it and not its neighbours has not been overlooked. Seed derived from self pollinated flowers of the fasciated plant are being grown to test this.

The sections of the buds were carefully examined for evidence of either insect or fungus action, and none was found. Such agents could not have been the direct cause of the flattening and forking which occurred in the sectioned part of the plant. Were the fasciation in this region due to such action, the stimulus must have persisted from an attack on an older part in a manner in which Knox (1908) believes it did in her *Oenothera* plants. One cannot entirely deny the possibility since evidence of the attack might not be apparent some months later, but no evidence was found.

It is remarkable that fasciation and branching should occur parallel with the ridge of the bed in which the plant grew (Fig. 1), and moreover that the plant should be more strongly developed on its north-west side from which the greatest amount of the autumn clearance was removed and as a consequence was subject to the greatest environmental change. One is thus driven to consider the possibility that the unilateral change in conditions was the primary cause of fasciation. Since, however, the change took place in a few minutes in the autumn and flattening and forking occurred repeatedly over a prolonged period running on in the sectioned buds to May and June of the following year, the change in conditions could not have been the immediate cause of each repetition. Persistence of the stimulus in some way or another must be postulated again.

It is possible to do no more than conjecture how such a one-sided change in the environment could bring about fasciation, but in the light of our growing knowledge of auxins such conjectures may perhaps be of interest.

It is now widely accepted that various responses to unilateral stimulation are due to a redistribution of auxin; unilateral illumination causes a decrease of auxin on the illuminated side and an increase on the 'dark' side. The autumn clearance must have subjected the plant to one-sided stimulation, and, what is more, this persisted till the following spring. During this time the plant was making little if any upward growth so that any auxin present in a particular part of the plant as a result of redistribution could not diffuse away to produce its normal effect, and it is not inconceivable that auxin 'trapped' in this way could cause increase in the tissues in its position of accumulation. Kraus, Brown, and Hamner (1936) have shown that cells may be caused to multiply by the external application of auxin, and Snow and Snow (1937) that the external application of auxin paste to stem apices results in an increased size, union, and displacement of leaf primordia. They say that the subsequent phyllotaxis is often permanently modified. Thus if we accept the possibility of auxin being trapped in the less strongly illuminated side of the stem, we may picture it here causing dilatation of tissues leading to flattening, and also to abnormality among leaves formed on that side leading to an irregular phyllotaxis.

The cause of bifurcation.

There have been few investigations into the forking which so commonly accompanies fasciation. Where broad fasciations have been concerned, fissure due to mechanical stress has been cited as the cause of forking (Knox, 1908; Bausor, 1937). Apart from this Knox apparently considers forking to be the primary effect, the fasciation being secondary. She says: 'once the physiological balance of the cells is changed and the chemical equilibrium altered by the peculiar stimulus of mechanical contact the tendency to multiply develops and frequently continues to the end of the life of the plant'. Worsdell (1905) regarded fasciation as a compromise between fusion and branching and infers that when normal growth of the plant is disturbed it reverts to an ancestral condition of dichotomy and pleotomy. Bausor (1937) seems to be following along these lines when he arranges his material in such a way as to show that sectors of cauliné nature are transformed into leaves and submits this in support of the homology of stems and leaves.

That physiological disturbance may result in fasciation accompanied by bifurcation is shown by Johnson (1926), who has produced such a condition in sunflowers, &c., by the action of X-rays. Snow and Snow (1937) link up effects such as these with results obtained by Skoog (1935) which go to show that X-rays destroy auxin and the implied suggestion of Laibach and Mai (1936) that division of embryonic parts may be brought about through local accumulations of auxin. They suggest that a decreased supply of auxin may be more readily divided into local accumulations.

If, as already suggested, the unilateral stimulation of the plant resulted in redistribution of auxin to the disadvantage of the stimulated side, the above might possibly explain the greater amount of forking on that side.

Whatever the physiological cause of the bifurcation may have been, there can be little doubt that it is structurally related to leaf formation. The presence of a leaf in each crutch of the axis (Fig. 6), the crossing over of the vascular strands in the bud sections (Fig. 7) and of the procambial strand in the flattened stem apex (Fig. 8), can be interpreted in no other way. One might even consider that the course of the crutch leaf bundle system was the cause of bifurcation.

When such a system is forming the vascular bundles commence to appear at the node and develop progressively upwards into the leaf and downwards into the stem, where they become connected with older vascular bundles, and I think we may say that connexion takes place with the nearest vascular bundle which is in a suitable state of development. When the fasciated stem becomes sufficiently flat it seems quite probable that a suitable vascular bundle on the opposite side of the stem may be nearer to the new one than its nearest neighbour on the same side. Connexion would then take place across the stele to divide it into two portions. These once instituted would form two channels of supply to the apex separated by a region of poor supply, if any, and the reasonable expectation would be the splitting of the apex into two.

Distribution of phyllotaxis irregularity and branching and repetition of flattening.

The plant suffers unilateral stimulation from the right-hand side which is presumed to result in concentration of auxin towards the left. The auxin 'trapped' on the left of the stem apex causes multiplication of the cells in that region and as a consequence flattening of the stem.

The localized accumulation of auxin also causes abnormal leaf formation on the left of the stem. As a consequence, since according to Snow and Snow (1937) each leaf forms in the next available space, the later formed leaves will be irregularly disposed. Leaves which are produced farthest from the position of initial abnormality one would expect to be least affected by it, so that the phyllotaxis on the right of the plant would be less irregular than that on the left. As leaves are formed higher up the axis, those above the more irregular region at the bottom of the left-hand side of the plant would be more irregularly placed than those above the right-hand more regular region. The left-hand branch of any pair was found to be the more irregular. Persistence of the unilateral stimulus would produce increasing irregularity so long as the upward growth of the stem apex were slow; when, however, elongation of the lower internodes carries the apex above the level of the unilateral conditions produced by the crest of the bed and the surrounding plants, unilateral stimulation ceases and we should expect the phyllotaxis to tend towards the normal. The observed fact is that irregularity waxes and then wanes.

Forking of the axis occurs when a leaf arises in such a position that owing to the flatness of the stem a procambial strand finds its nearest suitable connexion on the opposite side of the stele to that which bears the leaf with which it is associated. That there was a greater degree of branching on the right-

hand side of the plant may be due to the smaller amount of auxin present in that side rendering it more readily separable into local accumulations, together with the better conditions which prevail there for photosynthesis. This last may also explain why the right-hand side of the plant is taller and has thicker branches.

Provided the stem were sufficiently flattened, forking might be expected to reduce while not eliminating flatness. This was found to be the case by measurement of the plant. The increase of flatness which occurred, in other words the repetition of flattening, might be due in the lower parts of the plant to a persistence of the one-sided environment. In the upper parts, and possibly, too, in the lower, the increase of flattening might result indirectly from the already flattened shape of the stem. Given an initially flattened stem, any leaf borne on one of the flatter faces would subtend a stem sector with approximately parallel sides separated by a distance equal to the breadth of the leaf attachment, while a leaf arising on one of the more rounded edges of the stem would subtend a sector whose sides must, to allow for accommodation of neighbouring leaves, converge towards the inside of the stem. Thus the auxin diffusing through the stem from a leaf on the edge would presumably, on account of the smaller sectional area of its stem sector, be present in greater concentration and so induce greater cell multiplication at the edges with a consequent increase in flatness.

SUMMARY

The conditions of growth of a plant of *Digitalis purpurea L.* having a forked inflorescence axis are stated.

The abnormality is shown to be associated with fasciation and the morphology of the plant, including axis dimensions and phyllotaxis, is described.

A method of stating the irregularity of the phyllotaxis is evolved.

Descriptions of the course of vascular bundles and procambial strands in the forked stem apex are given.

The nature and cause of the flattening and forking of the stem are discussed in the light of previous work.

The microscopical part of this research has been aided by a grant from the Dixon Fund, University of London.

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Studies in Vernalisation of Cereals

V. The Inheritance of the Spring and Winter Habit in Hybrids of Petkus Rye

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With one Figure in the Text

INTRODUCTION

IN the course of a series of investigations on the vernalisation of cereals it was found that vernalisation of Petkus winter rye for fourteen weeks reduced the time required for flowering, and also the leaf number and tillering, to the value which holds for Petkus spring rye grown in the same day-length; longer treatment was without further effect (Purvis and Gregory, 1937). Since the characteristics mentioned serve to distinguish these varieties, it appears in effect that vernalisation converts a winter rye into a spring rye within one generation so far as physiological behaviour is concerned; in other words, it induces in winter rye a physiological condition leading to early flowering which is already inherent in spring rye. In a study of these conditions it is naturally of interest to ascertain which habit is dominant. In the literature the bulk of the evidence favours the dominance of the spring habit. Tschermak (1923), however, records that in wheat, but not in barley or in rye, the winter habit is dominant. It appears, further, that in wheat dominance of the winter habit is by no means general. Spillman (1909) obtained an intermediate type in the F_1 generation of a wheat cross and a $1 : 2 : 1$ segregation ratio in the F_2 , and similar results are recorded for various wheat and barley hybrids by Ollson, Schafer, McCall, and Hull (1920).

Nillson Ehle (1917) crossing Sol and Kolben wheats reports complete dominance of the spring habit, with a $3 : 1$ segregation in the F_2 generation, and Takahasi (1924) crossing a spring barley with three different winter forms obtained a $3 : 1$ segregation with the spring habit dominant in every case. Fruwirth (1923) states that this is the usual form of inheritance in barley. In a rye cross between Heinrich, a winter rye, and Sachsschen Standen a spring rye, Tschermak (1906) found complete spring dominance, and the segregation of the F_2 generation as a whole gave a fair approximation to the simple $3 : 1$ ratio; the direction of the original cross, however, affected

this ratio so that when the winter form was the seed parent the ratio of spring type to winter type was 4·6 : 1. Similar evidence will appear in this paper.

In a number of cases dominance of the spring habit is associated with less simple ratios. Cooper (1923) crossed two spring wheats with three winter varieties and found in all six cases that the spring habit was dominant in the F_1 generation. The segregation ratio in the F_2 generation, however, depended on which spring variety was used. With Marquis as spring parent the ratio was 13 : 3, while Manitoba 169 gave the simpler 3 : 1 ratio. Cooper's suggested explanation involves the assumption that the winter gene is dominant, but that its operation is suppressed by an inhibiting gene present in both the spring forms. This winter gene, he suggests, is present in the one spring variety, but absent from the other. Gaines (1917) gives a similar explanation of results he obtained from crossing two spring barleys, when he obtained approximately three winter type plants in every sixteen members of the F_2 generation.

Kajanus (1927), however, reviewing the genetics of wheat, considers that the genetic basis of the spring and winter types is by no means clear. He reports Cooper's results as an example of spring dominance and is not prepared to accept his explanation as recorded above.

The assumption that the winter gene is dominant but can be inhibited provides an explanation of the different types of inheritance described above. If W represents the winter gene and I the inhibitor, then the gametes of winter forms would have the genetic constitution Wi while those of spring forms might be wi , WI , or wI , and these, crossed with the winter forms, would give (1) 3 : 1 winter dominance, (2) 3 : 1 spring dominance, and (3) 13 : 3 spring dominance respectively. This does not, however, justify the statement that the winter *habit* is dominant. If the inhibiting gene has the effect of curtailing the long vegetative period associated with the 'winter' gene, then it is not clear on what grounds one may discriminate between such a gene and one carrying the spring habit. It would be interesting to compare the responses of spring varieties of different genetic composition to treatments such as anaerobiosis, which are known (Gregory and Purvis, 1937) to delay flowering.

This relatively simple inheritance in which one gene delays flowering beyond the minimum period, while a second gene may render the first inoperative, thus restoring the *status quo*, may possibly apply in a number of cases, but much more complex ratios, indicating the possible operation of multiple factors, have also been recorded. Vavilov and Kouznetzova (1921) in a wheat cross found 500 spring forms and 52 winter forms in the F_2 generation (reported thus by Cooper (1923) and by Aamodt (1923)). Aamodt (1923) also records the dominance of the spring habit in a cross between Marquis and Kanred wheats. His F_1 seed was sown in autumn, so that its earliness could not be assessed. The plants were harvested individually and 5,250 plants sown in the spring of the following year. Of these 4,808 eared before

autumn and 442 remained vegetative. It is perhaps significant that this apparently anomalous ratio (10·8 : 1) resembles that recorded by Vavilov and Kouznetzova (1921), i.e. 9·6 : 1. Among the 4,808 annual plants heading was spread over a period of eight weeks, the earliest flowering at the same time as the spring parent. Selected families were harvested from the groups heading in each week, and the F_3 plants grown separately from their seed. This revealed that the earliest group in the F_2 was homozygous, all giving spring plants, and the proportion of heterozygotes increased as the heading date of the F_2 parent became later. Moreover, some plants were homozygous for much later flowering-dates than the spring parent, and the late groups included plants which were homozygous for the winter habit. Aamodt regards his results as a demonstration of partial dominance of the spring habit, and considers that the operation of multiple factors, clearly indicated here, is responsible for the varied inheritance of the spring and winter habit in wheat.

Clearly there is no generalization which can be applied to all the cereals. Most of the evidence points to complete or partial dominance of the spring habit; indeed, Tschermak's wheat cross appears to be quite exceptional in this respect. Possibly in the diploid genus rye a single pair of factors is concerned, while in the tetraploid genera wheat and barley two or many pairs may operate. Since, so far as is known, no qualitative differences appear in the response of the temperate cereals to external physiological factors controlling flower production, it would appear probable that the genetic factors controlling flower formation are throughout identical, and therefore a general agreement in dominance might reasonably be expected. Until more is known of the actual physiological processes concerned in flowering and their relationship to genes, any attempt at explanation must be largely speculative. Melchers' (1937) experimental work on this aspect of the problem is discussed later. The inheritance of the spring and winter habits in the cereals would seem a fruitful field of study to the elucidation of which the work here reported contributes to some extent.

In the course of previous physiological work, when after suitable treatment the winter and the spring varieties of Petkus rye were simultaneously available, the opportunity was taken of attempting hybridization.

Owing to limited time and space the experiment was not carried beyond the F_2 generation. In addition, nothing is known of the genetic constitution of the two parents, and it is therefore impossible to determine exactly the nature of the inheritance, but it appears worth while to place on record a further contribution to the somewhat meagre data extant, especially as references to rye hybrids are few in number, a fact due no doubt to partial self-sterility in this species.

EXPERIMENTAL

In 1933 reciprocal crosses were made between Petkus winter rye and Petkus spring rye. In all, fifty-two grains of very variable size ripened and were

sown in sand culture pots on June 1, 1934. It may be noted here that in spite of the variation in grain size the final size of the plants varied little. About three-quarters of the grain was vernalised at 1° C. for 33 days, a period known to be short enough to allow the winter character, if dominant, to become manifest to a recognizable degree, yet long enough in such a case to ensure ripening before autumn. All the plants, however, whether vernalised or not, attained the anthesis stage in 43–45 days, while the spring parent planted on June 16 flowered 43 days after planting.¹ In flowering, tillering, and lack of response to vernalisation the hybrids showed a close resemblance to the spring parent; the dominance of the spring habit thus appears to be complete in the F₁ generation. Rye is partially self-sterile, with the result that covered ears produced little or no seed, and for this reason pollination was allowed to proceed freely among the F₁ plants, a course which was possible as no other rye was in flower at that time.

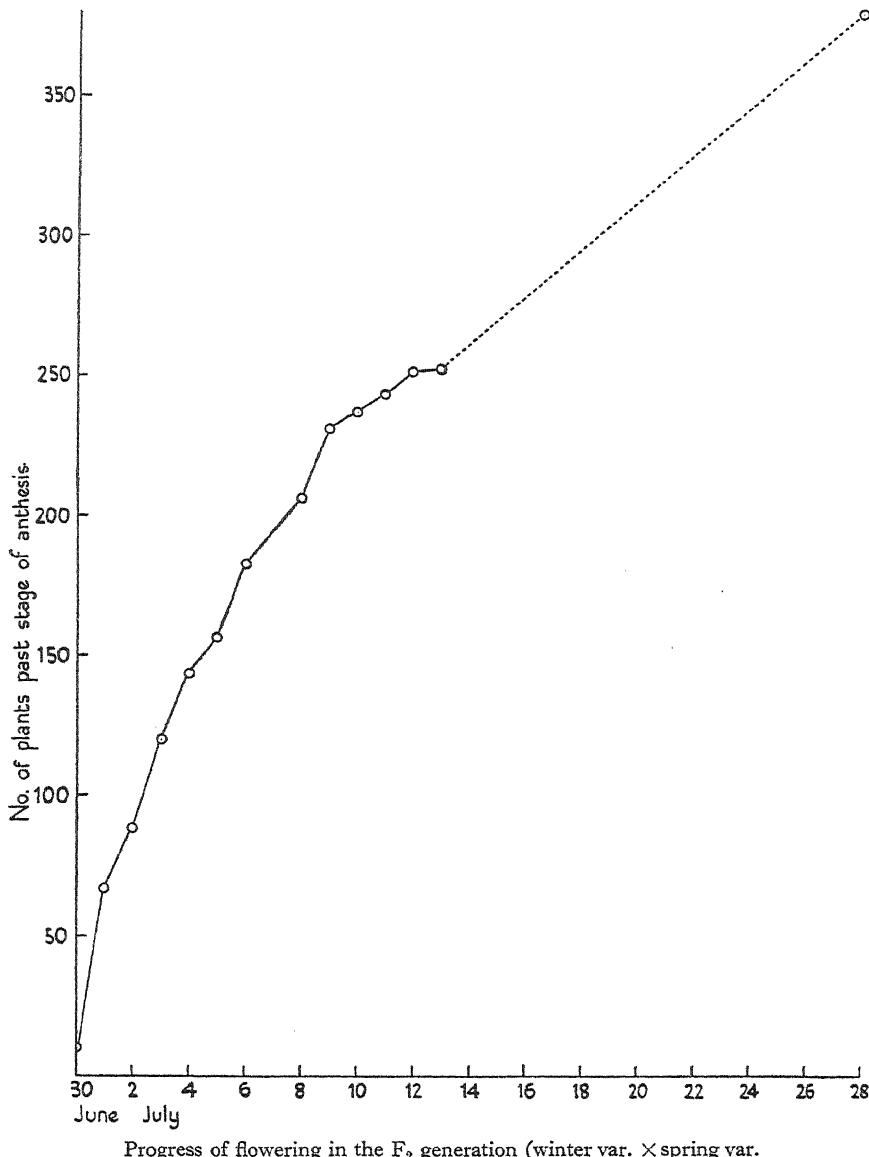
In the following year the seed from the reciprocal crosses was sown separately. The two original parent varieties were sown as controls on the same day as the F₂ seed (May 2).

Differences in tiller production became apparent on May 29, and 'shooting' was first observed in a number of plants on June 12. Flowering began on June 26 in hybrids and in spring controls, reaching a maximum in both during the first week in July. By July 28 nearly all the plants which showed any sign of shooting had flowered, and on August 15 these were dug up leaving a number of plants in the ground with the high tillering and rosette habit characteristic of spring-sown winter rye. The plants which set seed numbered 387, while among the remaining 134 were three plants with a few shooting ears. These three plants were all derived from the crosses in which winter rye was the seed parent; two of them were then flowering and one showed emerging ears, but as no seed eventually set, no test of their genetic constitution could be made. Spring-sown winter rye is known to flower sporadically in the autumn of the same year, requiring at least 150 days to reach anthesis, whereas these plants flowered 105–115 days after planting, thus more closely resembling spring types. On the other hand, their high tillering suggests that they belonged to the winter type. If these aberrant plants are included with the spring type the proportion of summer-flowering to over-wintering types found in the F₂ generation is 390/131, or 2.98 : 1, a close approximation to the ratio expected for a single pair of factors; if, however, they are classed with the winter type the ratio becomes 2.89 : 1.

In the F₂ generation, however, the dominance of the spring habit appears to be much less complete than in the F₁. Flowering in the spring control was nearly completed by July 6, while in the hybrid anthesis continued until late July, as shown in Table I.

If the progress of flowering is graphically presented (Text-fig.) it appears

¹ The term 'flowering' in this paper connotes anthesis in the most forward ear of a plant.



that the total number reaching this stage falls off considerably as July 13 is approached, suggesting that a second maximum must have occurred at some point subsequent to the middle of July to give the value attained by July 28. Unfortunately no data in this time interval are available.

This behaviour of the F_2 generation resembles that reported by Aamodt

TABLE I

Percentage of Plants in which Anthesis had occurred before the Dates shown

Date.	June 29.	July 6.	July 13.	July 27.	Total spring type.	Total winter type.	Ratio.	No. of plants.
W.R. ♀ parent	3·15	34·27	50·70	76·57	77·62	22·38	3·47/1	286
S.R. ♀ parent	1·05	36·84	48·95	70·53	70·53	29·47	2·39/1	190
Total of all hybrids	2·11	35·32	49·14	74·28	74·85	25·14	2·98/1	521
(These include a small number of plants not included in the above categories.)								
Spring parent	5·55	88·88	94·44	100	100	0·00		18

(1923), but the dispersion of flowering-dates is here very much less marked. The direction of the original cross appears to modify the ratio to some extent, crosses with winter rye as male parent producing a larger proportion of biennial plants, but since the discrepancy is not statistically significant it will require confirmation. Under the conditions of free pollination the effects of the original direction of crossing would tend to be eliminated, and this may possibly account for the lack of significance; it is realized that the flowers should have been self-pollinated, but as already stated, this leads to a high percentage of sterility. This influence of the direction of crossing has been noted by Tschermak (1909).

It was expected that in the late-flowering plants of the 'spring' type the winter character might be manifest in greater tillering as well as in delayed flowering. With the exception of the three very late plants referred to above, which did tiller somewhat more freely, this was not the case. In Table II it will be seen that late flowering is accompanied by a marked *reduction* in the number both of unripe tillers and of ripening ears.

TABLE II

Tillering in Relation to Flowering-date

Anthesis date.	Up to July 6.			July 6 to July 13.			July 13 to autumn.		
	Tillers per plant.	No. of plants.	Tillers per plant.	No. of plants.	Tillers per plant.	No. of plants.	Tillers per plant.	No. of plants.	Tillers per plant.
			Green.		Total.		Green.		
Hybrids	0·84	8·34	184	0·42	7·17	71	0·42	4·38	136
Spring parent control	2·00	8·56	16	1·00	9·00	1	0·00	3·00	1

Since the onset of flowering normally terminates tillering, this reduction indicates a low degree of vegetative vigour in the late-flowering plants, and this is further demonstrated by a high percentage of sterile or partially sterile ears among them as shown in Table III. 15·6 per cent. of the hybrids of annual type bore main ears with a few or no seeds, while this sterile condition did not appear at all in the spring controls; among 'early' hybrid plants (i.e. those which flowered before July 13), 6·6 per cent. bore main ears which were sterile in this sense, while among those-flowering after this date,

32·8 per cent. were sterile. The cause of this sterility is quite unknown, but in conjunction with the reduced tillering points to some lack of vigour. Sterility is slightly more frequent among plants derived from crosses in which winter rye was the seed parent.

TABLE III
Percentage Sterility in Relation to Flowering-date

Anthesis date	Up to July 13.	After July 13.	Total.
W.R. ♀ parent	7·59	37·66	18·02
S.R. ♀ parent	6·45	34·15	14·93
Total hybrids	6·64	32·83	15·64
Spring controls	0·00	0·00	0·00

The plants of winter type tillered very freely and over-wintered without loss in spite of their advanced state of development at the onset of winter. Flowering began early in the following June and was prolonged over a period of six or seven weeks. There was, moreover, considerable morphological variation, in height, in ear length, and in the nodding or erect habit of the ears. The sterility prevalent in the annual plants was absent in these. No attempt was made to continue the work beyond this point, but the winter type plants were allowed to pollinate themselves freely, and some of the seed from the earliest plants was used in another experiment (with due precaution against errors resulting from its variation) and gave rise to plants which behaved in all respects like winter rye.

DISCUSSION

The experimental cross here described establishes one more example of complete dominance of the spring habit in the first filial generation. The second filial generation, though giving a very close approximation to the expected ratio for a single pair of factors, shows divergences from the normal which require explanation. It is impossible to draw any definite conclusion on the slender data here presented, and it is hoped that the problem will soon be further investigated in this department, but meanwhile the possible significance of the results so far obtained is discussed.

The first question which arises is whether the dispersion of flowering-dates in the F_2 generation may be attributed (a) to an intermediate character in the heterozygous plants, (b) to the operation of multiple factors as in Aamodt's wheat cross (p. 720), or (c) to some other cause. The first explanation is not in accord with the clear-cut dominance in the F_1 generation, in which flowering of the whole population took place within three days, and simultaneously with that of the spring parent. Again, the dispersion of flowering-dates which occurred among the 'winter' type plants cannot thus be explained, and the late spring plants showed no sign of the prolific tillering which characterizes the winter type grown in conditions which prolong the vegetative period

(Purvis, 1934). On the other hand, the case does not appear to be analogous with that described by Aamodt, since the operation of multiple factors would not give a 3 : 1 ratio in the F₂ generation, or a dispersion of flowering-dates among the homozygous recessives.

A more satisfactory explanation may be that factors controlling early and late ripening during the one season segregate independently of those for the spring or winter habit. Tschermak (1923) lists early and late ripening as a pair of factors distinct from the annual or biennial habit; in wheat, as in barley and rye, early ripening is weakly dominant and appears to involve multiple factors. Harley and Martini (1929) obtained evidence for multiple factors in a very large number of crosses between early and late types of barley. The appearance of early and late individuals among both annuals and biennials thus becomes comprehensible, as does the absence of 'winter' tillering in the late spring plants. If this view be accepted, the control of the spring and winter habit in the two varieties by a single pair of factors is established, but little light is thrown on the inheritance of 'earliness' and 'lateness'. It is clear that 'earliness' is dominant. If it is the expression of a single gene, the following types should appear in the F₂ generation: 9 early spring, 3 late spring, 3 early winter, and 1 late winter; and there should appear among both annual and biennial plants, early and late individuals in the ratio of 3 : 1. In the case of the 'spring' plants there is some evidence of a second maximum (p. 723, Fig. 1) following a greater maximum in the first week of July. The dispersion about these two dates is, however, greater than the normal fluctuation found in spring rye. It would appear that several genes are concerned with 'earliness', each possibly associated with a different degree of earliness, or, it is tempting to suggest, with a different photoperiodic requirement, resulting in a sequence of flowering-dates through the changing day-lengths of early summer.

It is not possible to say which variety bears the 'early gene', but from experience in growing Petkus spring rye it is concluded that this variety is homozygous for earliness, since there is very little variation in flowering-date among plants sown at the same time, and since no still earlier phenotype appeared in the cross. In that case, winter rye must be either heterozygous or purely 'late'; this, however, involves the assumption that vernalisation treatment modifies both the 'winter' and 'late' habits, since by this treatment winter rye can be made to flower on the same date as spring rye (Purvis and Gregory, 1937). On this assumption it would appear that the 'spring' gene is similar in its physiological action to those for 'earliness', differing from them only in degree. If winter rye is regarded as heterozygous for 'earliness', light is thrown on the dispersion of flowering-dates which follows partial vernalisation, and which has been noted in an earlier paper (Purvis and Gregory, 1937, p. 573).

It is frequently stated by Russian exponents of vernalisation that there is no hard-and-fast line between spring and winter varieties of cereals; the

concept of independent genes for 'earliness' and for the spring habit make this statement more compatible with the clear 3 : 1 segregation of the spring and winter habit.

In this cross other factors appeared to be segregating—among the winter recessives variations in form were observed, while the late spring plants showed diminished vigour and increased sterility. It is possible that the latter may be explained in terms of the factors under consideration. Thus, winter plants carrying very early factors might flower in late summer, and so in conditions of temperature and day-length which prevent fruiting. The three plants which flowered in mid-August at least two weeks after all other flowering had ceased were possibly of this type, and their sterility may be due to short days or simply to the much reduced chance of cross pollination, since only three plants were then present. In these plants the high tillering indicates the 'winter' character. For similar reasons very late spring plants may have been unable to fruit freely. The restricted tillering of these plants, however, cannot be explained in terms of day-length.

The apparent, though insignificant, effect of the direction of the first cross demands consideration, in view of the fact that Tschermak (1906) reports a similar result. It may be significant that the crosses with winter rye as seed parent gave rise to the three August-flowering plants which on this hypothesis may have been very early winter types.

Clearly these considerations cannot be more than hypothetical, but the results do show that a careful genetical study on these lines would indicate the possible factors to be considered in the problem of flower production. In this connexion the work of Melchers' (1936-37) is of great importance. In the species *Hyoscyamus niger* he has confirmed the work of Correns (1904) that two races exist which are annual and biennial in their flowering behaviour. In these he has shown that the biennial race itself includes two forms, one of which may be vernalised, i.e. after low-temperature treatment it behaves like an annual. After spring sowing the annual type flowers during the summer while the other forms a rosette of leaves and a tuber, and flowers only after a winter rest. Correns found that hybrid plants formed rosettes, and yet began to shoot late in autumn. In certain respects, therefore, these races of *Hyoscyamus niger* resemble spring and winter forms of rye, but the biennial habit is dominant, though incompletely so. In the case of *Hyoscyamus* apparently, a single pair of genes was concerned.

Melchers has shown by grafting experiments that 'ripeness to flower' could be transferred from annual scions to biennial stocks, and that the potency of the annual forms in this respect was greater just before flowering. Thus it would appear that some substance, Cajlachjan's 'florigen' (1936), was transferred from the annual scion to the biennial stock. Since the biennial race flowers in its second year, and thus presumably itself produces florigen, the action of the scion may be due either (1) to a mere transfer of florigen to the stock or (2) to the earlier removal of the inhibiting action of

a gene in the stock which normally takes place only after a winter rest. Since low temperature can at any time during the first year's growth act in a similar way on the tissues of the biennial plant, this raises in the clearest way the question of the relation of the vernalising effect of low temperature to genetic constitution.

It would appear presumptuous for the present author to attempt an analysis of Melchers' data since his investigation is still in progress. It is significant, however, that Melchers (1939) has found it necessary to postulate a second hormone 'vernalin' to account for the specific effect of low temperature. Presumably, then, Melchers holds that two separate phenomena are concerned, associated perhaps with the 'early-late' and 'spring-winter' pairs of allelomorphs postulated above.

In conclusion, the author wishes to thank Prof. F. G. Gregory and especially Mr. F. Howarth for advice and criticism, and to acknowledge the co-operation of the staff at Chelsea Physic Garden where the work was carried out.

SUMMARY

1. Petkus winter rye crossed with Petkus spring rye gives a first filial generation in which the spring habit is completely dominant, while in the second filial generation 'spring' and 'winter' plants occur in approximately the ratio of 3 : 1.
2. In the F_2 generation within the 'spring' and 'winter' classes there is a dispersion of flowering-dates, suggesting a less simple inheritance.
3. Pending further investigation, this is explained on the basis of independent segregation of factors for early and late ripening as distinct from the spring and winter habit.

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Studies in Vernalisation of Cereals

VI. The Anatomical and Cytological Evidence for the Formation of Growth-promoting Substances in the Developing Grain of Rye

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With Plates XXII and XXIII and five Figures in the Text

INTRODUCTION

THE observations of Gregory and Purvis (1936, 1938) independently confirmed by Kostjučenka and Zarubailo (1938) that the vernalisation process in the Gramineae can be carried out on the developing embryo suggested a fresh investigation, by modern cytological methods, of the processes occurring in the embryo-sac of a cereal subsequent to fertilization. It was hoped to obtain by this means some clue to the functions of the various tissues of the developing fruit and to throw some light on the possible role of hormones in development. This reinvestigation has led to the discovery of new facts hitherto overlooked, more particularly the part played by the nucellar tissue in the organization of the mature grain and the sequence of degenerative changes associated with each phase of development.

To economize space previous observations which have been confirmed in this work are stated with the utmost brevity, citations of the relevant literature being given; only new observations are stated in detail. The data recorded are in part purely descriptive and in part quantitative; for clarity of presentation these two aspects are considered separately.

HISTORICAL SUMMARY

In the formation of the female gametophyte and in the development of the embryo, the grasses form a very homogeneous group and were the subject of repeated investigation by the earlier botanists. Hofmeister (1849) was the first to examine systematically the embryo-sac of the phanerogams, and he gives descriptions of a number of these, including among the monocotyledons, *Zea Mays* and *Sorghum bicolor*. In a later paper (1861) the same author included a partial description of the embryology of *Secale cereale*. This was later amplified in various particulars by Nörner (1881), Westermaier (1890),

[*Annals of Botany, N.S. Vol. III, No. 11, July 1939.*]

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Golinski (1893), &c. The scope of these contributions may be indicated very briefly.

Hofmeister (1861) in a general account of the Gramineae records for *Secale cereale* in particular the following observations. The outer integument of the mature ovule does not reach the micropyle, and the antipodals are very large and six to twelve in number. Only one embryo is found in each embryo-sac. This arises from the pro-embryo which first produces a club-shaped, undifferentiated mass of cells. Hofmeister also recognized the early, free-nuclear stage of endosperm formation; with regard to the rate of growth of the embryo of rye he states that from fertilization to the appearance of the first leaf primordium a period of only fourteen days elapses.

Nörner (1881) concerned himself with the earliest segmentations of the egg-cell of the cereal grasses, and the most advanced stage depicted would, from the data recorded in the present work, correspond with a stage nine days after fertilization. Three types of segmentation were distinguished, all of which have been found to occur in rye.

Westermaier (1890) studied the embryology of the phanerogams with particular reference to the antipodals and their possible functions, and included a description of *Secale cereale*. This work will again be referred to, but with the exception of new observations on the antipodals Westermaier added nothing of importance to the earlier descriptions.

Golinski (1893) was interested in the development of the anther and ovule; the part of his work which is of immediate concern is the very complete account of the behaviour of the antipodals after fertilization. The description Golinski gives of the mature ovule agrees so completely with the findings of the author that it would be superfluous to repeat it.

Among the monocotyledons, Hofmeister, Westermaier, &c., worked more particularly with the cultivated grasses, and since the details are very similar in all, it was possible for them to give a generalized account embracing all species. The same method of presentation was adopted by Schnarf (1929) in dealing with the embryology of the angiosperms as a whole; in his work the results of all previous investigators are brought together in an exhaustive generalized account.

Various authors, however, have dealt with the details of development of particular species. Thus Brenchley (1909), Modilevski (1938), and Percival (1921), have redescribed wheat, and Tannert (1905), oats. The differences among the cereals are sufficiently significant to have justified the separate investigations; moreover, advances in cytological technique in the interval have added even greater significance to these later descriptions. As far as can be ascertained a complete account of the development of the grain of rye has not yet appeared.

With the end which was in view in this investigation consecutive observations were made throughout the whole course of development, special attention being paid to the relations in time of the different phases, together with

their quantitative aspect. With the exception of the work of Modilevsky on wheat and occasional references elsewhere this aspect has not received adequate attention.

PART I

MATERIAL AND METHODS

A pure strain of rye, variety Petkus, was used for the investigation, both the winter and spring types being examined. The plants were grown in sand culture and were remarkably uniform in development: the main shoot of all plants flowering on the same day.

The method of collecting was as follows: The date of anthesis was marked for each ear on an attached label. Since Jost (1907) has shown that fertilization occurs in rye seven hours after pollination, this date was taken as the time of fertilization. The ears which were used to follow the development of the seed were collected at various intervals and the ovaries of the selected spikelets fixed.

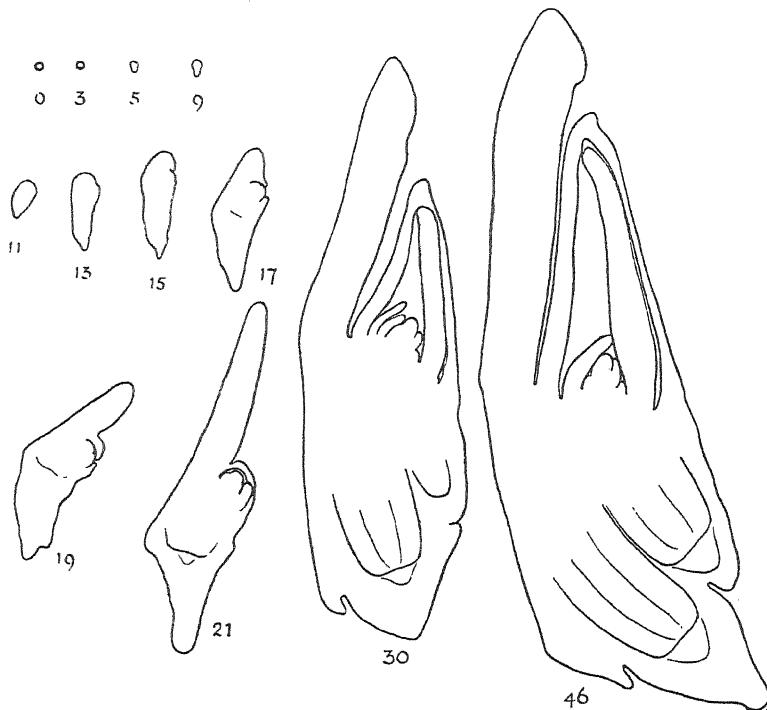
The first spikelet to flower occupies very generally a central position on the ear. On the date of anthesis only two spikelets (four flowers) on opposite sides of the ear may open: the remainder opening later, in succession, up and down the ear, at perhaps daily intervals, though this depends very much on the weather. Examination of the material shows also that fertilization does not invariably occur on the first day that the spikelet opens. The stigmas remain exerted so that pollination can occur at any subsequent date. It frequently happens, therefore, that the grain of any one ear may show stages of development differing from each other by as much as a week. To avoid errors in dating the material due to these habits of flowering only the four largest grains in any one ear were used.

Stages subsequent to fertilization were dissected from the spikelet, and the material incised to ensure better penetration of the fixing agents. A number of these were tried: Nawaschin's, Bouin's, Flemming's, and 2BE (La Cour, 1931); the last was found to be the most satisfactory. Fixation was, in all cases, effected under an air-pump. The material was taken up in the usual way through the alcohol series and alcohol-chloroform series into wax. The greater part of the material was cut at 10μ , using a Cambridge Rocker microtome. Owing to the early silicification of the fruit, more particularly the carpel wall and integuments, the material is difficult to cut, particularly the older stages. It has since been found that if the material is treated during dehydration with 10 per cent. hydrofluoric acid in 70 per cent. alcohol for forty-eight hours, this difficulty is largely overcome. (Chamberlain, 1932.)

Various staining techniques were employed: Newton's gentian violet method, alone or counter-stained with bismarck brown or orange G.; Flemming's triple stain and Heidenhain's haematoxylin and iron alum counter-stained with safranin, light green, &c. At some stages fresh material was hand-sectioned and subjected to a number of microchemical tests.

THE DEVELOPMENT OF THE EMBRYO

The zygote has no resting stage, but begins its development immediately. The first cell-division occurs on the second day, resulting usually in the division of the ovum into two equal segments by a horizontal wall. The



TEXT-FIG. 1. Median longitudinal sections of embryos at various stages of development. The figure beside each profile represents the age of the embryo in days.

variations of this behaviour and of the positions of the subsequent divisions have been dealt with by Nörner (1881).

Concomitantly with the early stage of development, the synergidae degenerate, as indicated by the more deeply stained appearance of the cytoplasm and the nucleus. By the second day after pollination the nuclear walls of both synergidae have disappeared, the chromatin remaining as deeply stained granules in the cytoplasm. The endosperm nuclei and the protoplasm of the embryo-sac in the immediate neighbourhood of the embryo share in the degeneration. The development of the embryo and the appearance of the embryonic organs as seen in median longitudinal section are shown in Text-fig. 1.

By the fifth day the embryo is known to comprise 16 cells and is roughly

pear-shaped. During the early stages of development the cell-walls laid down are predominantly anticlinal, not periclinal as figured by Nörner (1881), leading gradually to the formation of a definite dermatogen.

The embryo increases in size without change in form until about the eleventh day, when the first lateral primordium appears on the dorsal face of the embryo, i.e. on the side turned away from the stalk of the ovary, a little above its widest part. The cells in this neighbourhood show denser contents and have larger nuclei, and take on the appearance of a meristem as distinct from the rest of the embryo. This constitutes the stem apex and projects very slightly as an added convexity to that of the remaining surface.

On about the fourteenth day after fertilization a crescentic ridge—the coleoptile primordium—appears along the upper margin of the stem apex. This extends both around and over the meristem until the latter is completely enclosed except for a small pore, which is lateral in position with reference to the meristem, but on the median dorsal line of the whole embryo.

The primordia of the first, second, and third foliage leaves now appear at approximately weekly intervals and have a divergence of $1/2$ with respect to each other and to the coleoptile primordium. Each leaf begins as a crescentic ridge and then grows to enclose the meristem and the younger leaves in a conical cap. The edges of the cap do not fuse but at a later stage become infolded. The whole cap represents the leaf-blade, the leaf-sheath being absent in the embryo.

The leaves differ in development from the coleoptile in that the latter completely encloses the younger parts of the plumule except for the small pore already mentioned; whereas the leaf-blade is open along nearly its whole length. This pore in the coleoptile has a special position in that the first leaf primordium develops exactly opposite it and at first protrudes somewhat through it (Text-fig. 1, days 17 and 19), when it is seen that the immature aleurone layer is now in contact with the first leaf primordium and is degenerating rapidly in its neighbourhood.

The coleoptile and leaves differ also in their vascular structures. In the former, two vascular bundles can be traced, which are exactly similar, diametrically opposite each other, and lateral in position. They do not affect the external morphology of the coleoptile. Several vascular bundles, on the other hand, occur in the embryonic leaves. These are parallel and unbranched, and their positions are marked by vertical ridges on the adaxial (upper) surface of the leaf.

Of the three foliage leaves found in the mature embryo, the second is but a quarter the length of the first, and the third is present only as a ridge on the meristem. The plumule of the mature embryo also bears in the axils of the coleoptile and first leaf the primordia of the first and second tillers respectively.

The first indication of root development appears at about the same time as the differentiation of the stem apex as a special orientation of cell division

in the centre of the embryo, slightly below the level of the plumule. In this region chains of cells radiate in every direction except towards the basal end of the embryo. These new cells separate from the adjacent tissues of the embryo, thus constituting a schizogenous cavity which is first in the form of a transversely orientated disk which later becomes cup-shaped, thus delimiting the coleorhiza from the root. The original space between the early root primordium and coleorhiza quickly becomes filled by the well-defined root-cap of the primary radicle.

The zones of the root, dermatogen, periblem, and plerome, are clearly distinguished both in longitudinal and transverse sections. The central cell row of the plerome is notable in that it is composed of particularly large disk-shaped cells. The cortex of the root is traversed by longitudinal air spaces, which in transverse section appear as circular or trigonal areas of considerable extent: sufficient almost to define the cortex as an aerenchyma. The cells of the dermatogen are columnar, thus resembling those of the epithelium of the scutellum. Cell remnants in the cavity between dermatogen and coleorhiza may indicate also that a breakdown of the coleorhiza tissue occurs. The primary root goes on elongating until the embryo reaches maturity. Meanwhile four further roots arise in a way similar to that of the primary root. The second root arises in the median plane of the embryo, above the level of the first and in a ventral position. The third and fourth roots appear in succession laterally, on the opposite sides of a medial line defined by the positions of the first and second roots. Finally the fifth root occupies an asymmetric position between the second and the fourth, on the ventral side of the embryo. At maturity the five roots lie close together, separated only by the disorganized remains of the cells of the coleorhiza. The positions of the apices of the secondary roots are marked externally by downwardly directed processes of the surface of the coleorhiza. These bear the same relation to the secondary roots as does the rest of the coleorhiza to the primary root. No epiblast can be distinguished.

The scutellum arises as a growth of the dorsal surface of the embryo into a shield-shaped structure. The extension is chiefly in a distal direction—since the plumule of the embryo remains in a lateral position—and is effected by the activity of an apical meristem. The lateral edges of the scutellum grow over the sides of the coleorhiza, mesocotyl, and coleoptile.

The epidermis of the scutellum is an epithelium composed of cells very much elongated in an anticlinal direction and regularly polygonal in surface view. During the growth of the embryo the endosperm cells in contact with the epithelium degenerate and become absorbed. Brown and Morris (1890) have described the secretion of diastase and cytase by the epithelium of the scutellum of germinating barley, and it is probable that similar enzyme systems are concerned in the dissolution of the endosperm before maturity. When the embryo has attained its full size the process ceases, and the epithelium now abuts on the so-called 'depleted layer' of the endosperm, which

consists of a narrow stratified zone of the compressed cell-walls of the exhausted tissue, containing nuclear remnants.

A central vascular bundle can be traced from the mesocotyl region nearly to the tip of the scutellum, where it terminates as a number of fine separating traces. The interpretation of the morphological relationship of the coleoptile, scutellum, and coleorhiza and the comparative morphology and anatomy of the grass embryos in general have been dealt with by Bruns (1892), Celakovsky (1897), Van Tieghem (1897), Sargent and Arber (1915), Avery (1930), Arber (1934), as well as others.

THE ENDOSPERM

Immediately after fertilization the triple fusion nucleus repeatedly divides to produce a very large number of nuclei lying free in the embryo-sac and not separated by cell-walls. These nuclei occupy a peripheral position in a lining layer of cytoplasm and are more abundant on the ventral side of the embryo-sac, in which region also the lining layer of protoplasm is thicker. On the opposite side of the embryo-sac there is only a single layer of triploid nuclei connected by thin protoplasmic strands. The narrow non-vacuolate micropylar end of the embryo-sac contains dense protoplasm and numerous endosperm nuclei which may surround the developing embryo and degenerate in its neighbourhood. In the remainder of the embryo-sac the vacuole is in process of very rapid extension during the free nuclear stage of endosperm formation. Since the embryo-sac occupies a very large part of the total volume of the ovary this increase in size is correlated with a rapid expansion of the whole fruit. Changes in growth-rate of the embryo-sac will be dealt with later.

The endosperm remains 'free nuclear' until the conclusion of the most rapid stage of embryo-sac extension, i.e. until the sixth day. The subsequent segmentation occurs by the centripetal formation of cell-walls and begins in the neighbourhood of the ventral groove. This is complete eight days after fertilization. It is not uncommon to find that at the termination of cell-wall formation some free nuclei remain in the centre of the embryo-sac which later become compressed by the developing endosperm and eventually disappear.

The extension of the embryo-sac is mostly in a longitudinal direction, and during this the fruit assumes its characteristic elongated form. In transverse section the endosperm is kidney-shaped and half divided by the ventral groove into two parts. Following the terms used by Brenchley (1909) in describing the grain of wheat, the lateral parts of the endosperm will be referred to as the 'flanks' and the median portion opposite the ventral groove, the 'bridge'.

When first segmented the cells of the endosperm are large and vacuolate; but further nuclear divisions have been observed after this stage. The nuclei are prominent and contain three nucleoli. The formation of starch in the endosperm does not begin until this segmentation is complete, and is first observed in the centre of the flanks at a position three-quarters the length of the fruit from the proximal end. From these points accumulation of starch

proceeds in both directions along the length of the endosperm, to the margins of the flanks and finally in the region of the bridge. The details of starch formation in rye are similar to those in wheat, as described by Brenchley (1909), the quantitative aspects of the chemical changes occurring in rye endosperm have been recorded by Müntz (1878).

At the time of first formation the peripheral cells of the endosperm are indistinguishable from the remainder except that they are slightly smaller. About four days later, i.e. at ten days, however, the periphery becomes differentiated as the aleurone layer. Notably in the region of the ventral groove the cells of this layer attain denser protoplasmic contents than the neighbouring starch-storing parenchyma, and here the differentiation of the aleurone layer is accompanied by almost complete digestion of the remaining nucellar tissue. It is only the cuticularized epidermis of the nucellus and a narrow column of cells of the raphe that remain intact. In the former case the contents of the cells disappear, whereas in the latter the cells become occluded with resinous materials.

Pl. XXII, Fig. 1, shows a camera lucida drawing of a median longitudinal section of the grain in the region of the ventral groove, at an early stage of the dissolution of the nucellar tissue. It can be seen that the cells of the aleurone layer have the appearance of a secretory epithelium, and that the dissolution of the nucellar tissue takes place in a way similar to that of the endosperm in contact with the epithelium of the scutellum, a compressed stratified layer of fragmented cell-walls alone remaining.

Aleurone grains in the aleurone layer show themselves at an early stage, and the accumulation is most rapid and complete in the ventral portions of the flanks, and least along the ventral groove. During this storage of protein the cells become more regularly rectangular in longitudinal section, and cell-walls thicker. The mature aleurone layer cell is completely filled with aleurone grains of uniform size which do not, however, distort the nucleus by their mutual pressure. The aleurone layer differs from the rest of the triploid tissue in that it remains alive throughout the period of dormancy and becomes active again on germination.

THE ANTIPODALS

The changes occurring after fertilization in the antipodal cells comprise a very striking increase in size, a simultaneous multiplication and increase in size of nuclei, followed by rapid and early degeneration. These processes are of great interest in the development of the grain as a whole, and it is proposed to deal with them in detail.

There appears to be no definite number of antipodals in the mature ovule. Thus for rye, Hofmeister (1861) mentions six to twelve, Golinski (1893) eight to twelve cells and twelve to eighteen nuclei, and Westermier (1890), and Lütscher (1905), 'numerous' antipodals. In the present material a minimum number of six antipodal cells can be counted at a stage just previous to

fertilization; of these, three cells are uninucleate, two are binucleate, and one trinucleate, comprising a total of ten nuclei in all. As a maximum in the unfertilized mature embryo-sac eighteen nuclei have been counted.

At earlier stages of development the antipodal cells are non-vacuolate and contain dense protoplasm; they become vacuolate by the time of fertilization and extend into the embryo-sac as a loose tissue attached to the chalazal end, being therefore lateral. This position is maintained, and as they participate in the general extension of the embryo-sac they are carried farther away from the micropylar end. At the time of their formation the antipodal nuclei are of considerable size but no larger than those of the synergidae and ovum. The subsequent increase in size is shown in Pl. XXIII, Figs. 4–6. Thus, at the stage just mentioned, a typical diameter is 10μ . At fertilization this has increased to 36μ : and just previous to disintegration the nucleus may have a diameter of 54μ . These figures may be compared with those of Beck et al. (1932) for the size of the antipodal nuclei of *Bromus* spp. Here a diameter of $11\mu \times 15\mu$ at fertilization increases to a maximum of $55\mu \times 36\mu$ and a minimum of $33\mu \times 26\mu$ shortly afterwards. While they are increasing in size the antipodal nuclei undergo multiplication: thus the largest number of antipodals observed is 33 in spring rye and 26 in winter rye, three days after fertilization; while on the previous day the largest number counted is 23. Between the third day and the sixth day the antipodals degenerate, and this is shown by the fewer number counted during this period; up to nineteen may be counted on the fourth day after fertilization and fourteen on the following day. Unfortunately insufficient material was collected to compare the number of antipodal nuclei in the spring and winter varieties. After this stage it is difficult to identify the fragments as separate nuclei and further counts are not possible. The actual data are listed below in Table I.

TABLE I
Number of Antipodal Nuclei

Stage.	Winter rye.	Spring rye.
Before fertilization	10	—
At fertilization	18	14+, 17
1 day after fertilization	19	—
2 days	22, 23	—
3 „ „ „	16+, 26	33, 26
4 „ „ „	19	15
5 „ „ „	14	Fragments only
6 „ „ „	Fragments only	—

It is impossible to discover from the material whether this increase in number of nuclei is accompanied by a corresponding increase in number of antipodal cells. At the earliest stages the antipodal cell-walls are extremely delicate and during the expansion of the cells they become more and more attenuated and difficult to identify. Owing to the large size of the cells during the time the nuclei are observed to increase in number it is unlikely that

cell-walls are laid down, and the known fact that antipodal cells before fertilization are multinucleate makes it probable that no subsequent cell-wall formation occurs. In the later stages the antipodal cell-walls are most clearly seen in sections of fresh material stained in Sudan III. The staining reaction, though faint, is sufficient to suggest that the cell-walls may be cuticularized. This confirms the observations of Osterwalder (1898) and others on the cuticularization of the antipodal cell-walls in other families. Once the nuclei have begun to degenerate the cell-walls can no longer be distinguished.

The giant antipodal nuclei of the Gramineae and of the Ranunculaceae have attracted the attention of cytologists, and before describing in detail the changes which occur in these nuclei during their growth and degeneration, the observations of earlier authors will be considered. Previous to the work of Golinski (1893) no attention was paid to the internal structure of antipodal nuclei. This author describes in detail the changes observed in the nuclei in rye, and figures chromatin of the giant nuclei as twelve spherical granules arranged in pairs. He suggests that this arrangement indicates the division of six original granules into twelve. One, two, or three nucleoli, often 'crystalline' in form, are described as containing a number of lighter 'endo-nucleoli' which increase in size in the later stages, change from spherical to an elliptical shape, and are stratified like starch grains. More recently Beck et al. (1932) describes the chromatin of the giant antipodal nuclei of *Bromus* spp. as 'rope-like' and the nucleolus as vesicular. Of interest in this connexion are the observations of Osterwalder (1898) and Huss (1906) on various species of Ranunculaceae. The former author describes the chromatin of the giant antipodal nuclei of *Aconitum Nappelus* as in 'clumps', which, in the degenerating nucleus, break up into 'shapeless chromatin threads'. Recently Jachimsky (1937) has re-examined this example as well as other species of *Aconitum* and *Podophyllum peltatum*. He summarily rejects Osterwalder's descriptions as inaccurate, and states that, on closer examination, the balls and clumps are seen to consist of a dense reticulum of anuclear threads bearing granules of chromatin. This author also portrays the nucleolus as containing large and small vacuoles as well as bodies of chromatin connected to the external reticulum by a thread of anuclear material.

The examination of the present material shows that the antipodal nuclei of the Gramineae resemble closely those of the Ranunculaceae. Before the embryo-sac is mature the antipodal nuclei have the normal appearance of resting nuclei. The reticulum is uniformly distributed and of an even texture (Pl. XXIII, Fig. 3). There is present a single large nucleolus and about seven smaller deeply stained granules in scattered peripheral positions. During the rapid expansion of the nucleus the reticulum becomes extended so that the whole appears less deeply stained (Pl. XXIII, Fig. 4); at the same time the reticulum tends to become concentrated in special regions, leaving parts of the nucleus completely free of any chromatin material. This condensation is continued until the stage represented by Pl. XXIII, Fig. 5 is reached. There is

no doubt that this is the appearance of the chromatin described by Osterwalder as in 'clumps', and careful examination confirms the observations of Jachimsky that these masses have a reticulate structure, and are not solid particles of chromatin as Osterwalder believed. However, there is evidence to indicate that this condensation of the reticulum takes place about the granules observed in the early stages. The number of the original granules and of the final, large concentrations of chromatin is equal to the haploid number for the species. The nuclei are, of course, haploid, and it is reasonable to conclude that these clumps represent the condensed reticula of the seven haploid chromosomes. The clumps are widely separated, in this resembling the separation of bivalents in diakinesis; superficially at this stage the nuclei have the appearance of meiosis. Here, however, only single chromosomes constitute each clump, and these are not condensed in length but still compose an anastomosing network.

The division of six original granules into twelve as described by Golinski (*loc. cit.*) has not been observed, and it is difficult to determine whether this description concerns the seven original granules or the seven final clumps of chromatin. These granules, which are found in association in later stages with the condensed reticula, increase in size, remain globose and, with the nucleolus, persist after the disappearance of the chromatin.

The nucleolus increases greatly in size during the expansion of the nucleus, and changes in form from spherical to kidney-shaped. There are indications that chromatin bodies occur inside the nucleolus, and the presence of vacuoles (the 'endonucleoli' of Golinski) is confirmed. The latter increase in size but as far as can be observed are not stratified and do not change in form.

The degeneration of the nucleus, once the maximum size has been reached, begins by a breakdown of the nuclear wall (Pl. XXIII, Figs. 5 and 6). The clumps of chromatin, now released into the plasma of the embryo-sac, retain their individuality only for a short time, stain irregularly and more deeply, and then disappear. The nucleoli and the globose granules lose their staining reaction and can be identified as the final remnants of the antipodal nuclei (Pl. XXII, Fig. 2 A.).

Pl. XXIII, Figs. 5 and 6, show that the growth and degeneration of the antipodal nuclei are not simultaneous but successive. This is also indicated by the nuclear counts.

THE NUCELLUS, INTEGUMENTS, AND CARPEL WALL

In the mature fruit four separate tissues enveloping the endosperm and embryo can be identified. These are the same structures which enclose the embryo-sac of the mature ovule, i.e. the nucellus, inner integument, outer integument, and carpel wall; they undergo a new development simultaneously with that of the embryo-sac. The discontinuous growth of the latter is simulated by the growth of the various envelopes.

Thus at the early stage of the most rapid extension of the embryo-sac the

distal parts of the enclosing cell-layers elongate by extension growth; and the subsequent less rapid elongation of the embryo-sac is then accompanied by the development of all the integuments of the fruit by means of meristematic zones in their basal halves. These intercalary meristems not only enlarge the nucellus, integuments, and carpel wall in a distal direction, but also provide for the deepening of the narrow micropylar region of the embryo-sac that is a prominent feature of the earlier stage of development of the fruit (Text-fig. 2, *d*, *e*).

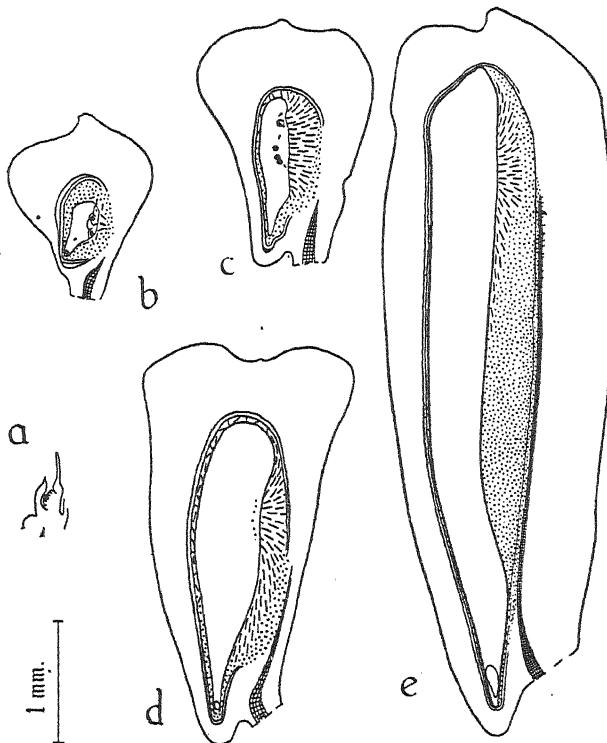
During these processes the various cell-layers enclosing the endosperm and embryo, in general retain the characteristics found at the time of fertilization. The structure and development of the analogous cell-layers in wheat have been dealt with in great detail by Percival (1921), whose description applies equally to rye.

The carpel wall is at all stages composed of larger cells than the integuments, and its growth proceeds more by elongation than cell division, also, in contrast with the nucellus and integuments, it contains abundant starch grains. At the later stages of the drying down of the fruit the carpel wall shrivels and becomes the wrinkled, papery pericarp, which can be easily removed; in this it differs from the integuments which are closely adherent to the grain.

The development of the nucellus after fertilization proceeds in a way similar to that of the integuments and carpel wall, namely by the cell extension in early stages followed by intercalary growth from the base; this tissue, however, undergoes important new developments. At the time of fertilization the nucellus consists of a considerable tissue ventral to the embryo-sac and a rather thin layer elsewhere. Except at the chalaza it breaks down early in the extension of the ovary, until the embryo-sac is limited laterally and dorsally only by the persistent epidermis of the nucellus. The extension growth of the nucellus is shown in Text-fig. 2. The directions of the long axes of the extended cells are indicated in the diagram, and the regions in which the cells remain isodiametric and meristematic are stippled. It will be clearly seen that during this stage of development these lines of extension converge to a point on the boundary of the nucellus in the region of the degenerating antipodal. The stage represented by Text-fig. 2, *d*, is shown in detail in Pl. XXII, Fig. 2, which is a longitudinal section of the extending nucellus. Golinski (1893) also noted that the nucellus extends towards the antipodal.

At the same time the cells in the neighbourhood of the end of the vascular bundle of the raphe become meristematic and begin rapid division. This zone of rapidly dividing cells constitutes an intercalary meristem which provides for the further growth of the nucellus. As a result of this activity the nucellus in the region of the ventral groove develops at the same rate as the rest of the ovary and eventually forms a column of tissue occupying a median position in the fruit. This will henceforth be referred to as the 'nucellar pillar'; on reaching its maximum development it is composed of a compact tissue of isodiametric small cells, containing no intercellular spaces, and is the largest

single organ of the fruit at this time. After the stage represented by Text-fig. 2, *d*, no further extension growth of the nucellus takes place, and at a later stage of development of the nucellar pillar the original extended part can still be identified at the distal end of the ovary (Text-fig. 2, *e*).



TEXT-FIG. 2. Median longitudinal sections of developing grain to show the extension growth of the embryo-sac. The directions of extension of the cells of the nucellus are indicated by hatched lines, the meristematic regions are stippled, and the vascular trace of the raphe cross-hatched. *a*, The carpel at the time of the formation of the megasporo母-cell; *b*, at fertilization; *c*, two days after fertilization; *d*, four days after fertilization; *e*, six days after fertilization.

The vascular bundle of the mature ovary before fertilization terminates at the base of the raphe. By the growth and differentiation of the carpel wall in this region, the vascular bundle extends along the raphe concomitantly with the development of the nucellar pillar. Thus the fully elongated fruit contains a vascular trace which traverses almost its entire length. In cross-section this is found to consist of a ring of three or four groups of vascular elements, the median, adaxial one being the most prominent.

The remarkable and almost total disappearance of this nucellar pillar has been described when dealing with the development of the aleurone layer. In

the hard ripe grain only a narrow strand of the nucellar pillar occluded with resinous materials remain. The behaviour of the cuticularized epidermis is entirely different from the rest of the nucellus. Except at the ventral groove it constitutes the single cell layer of nucellar tissue bounding the embryo-sac, and develops with the growth of the latter. It is composed of large cubical or oblong cells containing large nuclei, and in general has the appearance of a tapetum. At the time of the dissolution of the nucellar pillar the cuticularized epidermis resists the disintegrating action of the aleurone layer. This is particularly conspicuous in the vicinity of the ventral groove, where the epidermis of the nucellus remains intact, whereas the underlying cells in contact with it are completely digested. Here, where the most evident dissolution is proceeding, the radial walls of the epidermis of the nucellus are also cuticularized, and it is probable that this in some way protects the tissue from the secretion of the aleurone layer. The cells of the epidermis of the nucellus lose their contents but for some time retain their shape; it is only in the final stages of the drying down of the grain that they become compressed between the aleurone layer and inner integument.

PART II

QUANTITATIVE DATA ON THE GROWTH OF EMBRYO AND EMBRYO-SAC

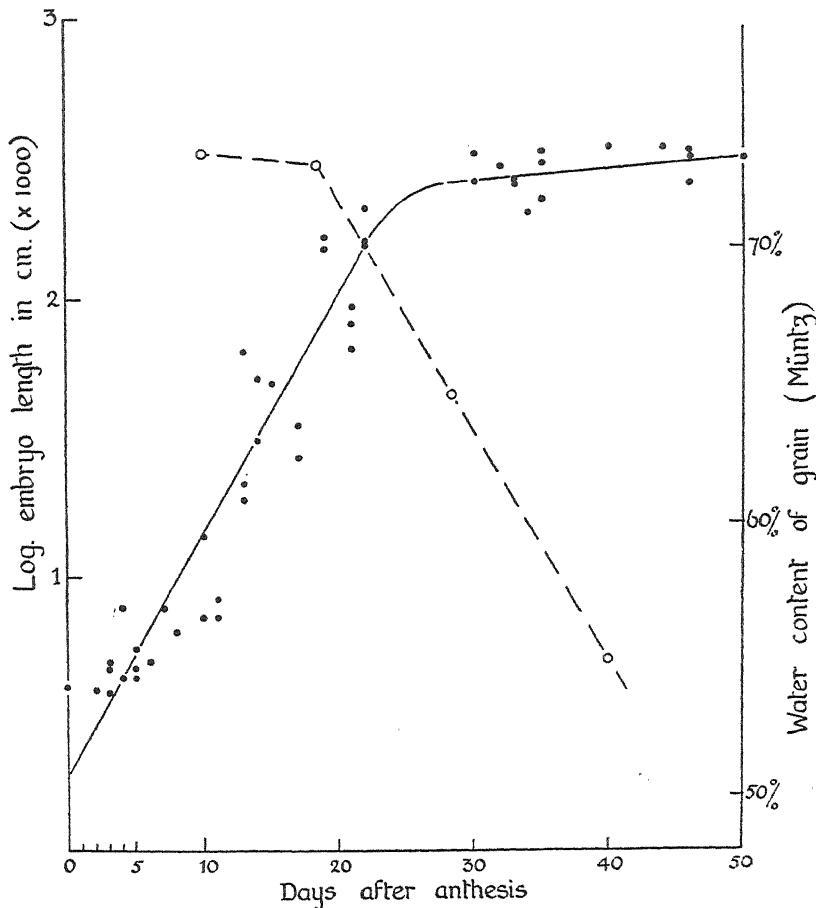
From the material gathered at varying known periods after fertilization, as described above, the length of embryo and embryo-sac were measured with a micrometer eyepiece in median longitudinal sections. In the early stages of growth of the embryo it was possible to observe directly the number of cells formed, and it was found that up to six days divisions occurred regularly, thus doubling each twenty-four hours the number of cells. Although it was not possible to carry observations beyond this point, yet the total number of cells in the mature embryo was determined in the following way. From serial microtome sections of the mature embryo a wax model was constructed from which, by weighing, the total volume of the embryo was determined. The volume thus found was 1.89 cu. mm. In regions of the sections corresponding with the various tissues the total number of nuclei in a given area was counted. As the thickness to which the material was cut was known, it was thus possible to estimate the number of cells per unit volume and also to determine the volume of each tissue. For various tissues the number of nuclei in a square field of side 50μ in sections 10μ thick are shown in Table II below.

TABLE II

No. of Nuclei in a Field of Volume $50\mu \times 50\mu \times 10\mu$

Region of embryo.	No. of nuclei.
Scutellum	6
Stem apex	24
Root	14.4
Coleorhiza	6.5

In this way the total number of cells in the mature embryo was estimated as 1.2×10^6 . Assuming that the nuclei double each day, as observed in the early stages, throughout growth of the embryo, it is clear that the number

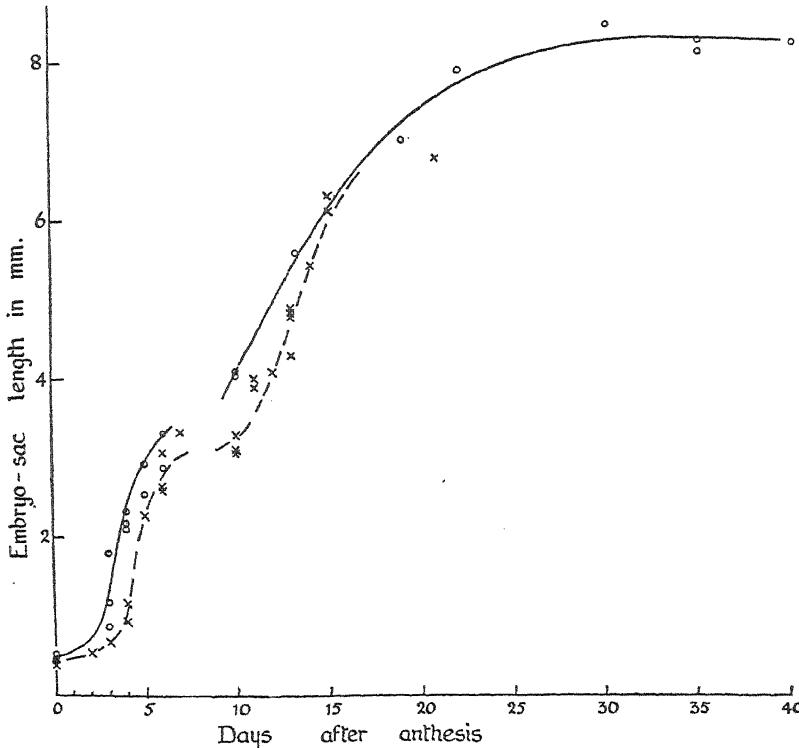


TEXT-FIG. 3. Rate of growth of the embryo. Solid line=relative growth-rate. Dotted line=percentage water content.

of days required to reach this figure is given by $2^n = 1.2 \times 10^6$, where n is one less than the total number of days. The number of days required is thus found to be approximately twenty-one. The calculated value may be compared with the course of growth in length of the embryo as given in Text-fig. 3.

In the figure the logarithm of embryo length is plotted against the number of days from fertilization. In this diagram the values of spring and winter rye are not separated; no consistent difference could be shown, and therefore all data are presented together. It is seen that the curve has two well-defined

limbs. Up to approximately twenty-two days from anthesis the relative growth-rate of the embryo is constant, during which time the growth approximates very closely to an exponential mode: while after this practically no further growth occurs. The value noted above, during which there is



TEXT-FIG. 4. Rate of growth of the embryo-sac (circles=spring rye; crosses=winter rye).

regular multiplication of cells, is thus found to agree very closely with the period of exponential growth seen in the figure. It may therefore be concluded with considerable probability that cell-division occurs throughout the embryo each day until nearly maximum size is reached.

The cessation of growth noted apparently coincides very closely with the onset of desiccation of the fruit. The data obtained by Müntz (1878) for the rye grain showing the water content at varying times after fertilization are inserted as a dotted line in Text-fig. 3 to bring out this point.

A further deduction may be made from Text-fig. 3. It will be seen that the curve cuts the ordinate at a value corresponding with a length of the embryo at fertilization of 19μ . By direct measurement cell size in the meristematic apex was found to be exactly this value, which confirms in a striking way the deductions made above.

In Text-fig. 4 the growth in length (mm.) of the embryo-sac is shown. The extension growth is markedly different from that of the embryo itself in two respects. Firstly, a difference appears between spring and winter varieties which, for this reason, are separately represented in the diagram. The spring rye has a more rapid extension growth, but final length is the same in both varieties. Secondly, extension growth of the embryo-sac shows two independent cycles, the first occupying approximately 8 days. The curve for this cycle is seen to be sigmoid in form, showing a very rapid increase up to four days and a marked decline during the next four days. Apparently a period of cessation or a very slow growth now occurs, but growth is resumed at ten days from anthesis with a high initial rate, falling approximately logarithmically until maximum size of the embryo-sac is attained.

Such a discontinuous growth has been noted in the pericarp of the cherry by Tukey (1933). Here also the same stages may be distinguished, i.e. rapid preliminary and final phases separated by a period of slow growth. The preliminary stage of rapid extension here occurs first in the pericarp, followed by growth of nucellus, integuments and embryo-sac. During the intercalated period of slow growth of the pericarp, the embryo and endosperm undergo their most rapid enlargement, and it is not until these tissues are approaching their final size that the second phase of extension growth of the pericarp supervenes.

In the case of the rye grain the growth of the fruit resembles in some respects that of the cherry as studied by Tukey. Each stage in the growth of the embryo-sac is directly correlated with some important development occurring within the embryo-sac. Thus the very slow initial stages of extension before three days coincide with the multiplication of the antipodal nuclei; the very rapid growth now following covers a period during which the antipodals degenerate. Meanwhile the localised extension growth of the nucellar tissue is taking place, as already described, and represented in Text-fig. 2. The slowing down of this phase corresponds with the rapid multiplication of the endosperm nuclei and the organization of the endosperm tissue.

When growth of the embryo-sac again begins, the nucellar pillar is formed at the same time; development also takes place in the carpel wall leading to the differentiation of the vascular supply. After ten days the aleurone layer is rapidly forming and soon invasion and dissolution of the nucellar tissue begins. The embryo meanwhile increases by constant exponential growth, and is complete at about the same time as the embryo-sac has reached the end of its second growth phase. The final stages of this extension marks the period of rapid starch accumulation in the endosperm and the beginning of desiccation.

DISCUSSION

From the data already presented it is possible to co-ordinate the development of the embryo, embryo-sac, and tissues of the fruit, and to assign to

the various stages their duration. It is then seen that the initiation of each new phase of development occurs by the degeneration of some previously formed tissue in the embryo-sac. A summary of these relations is given in Table III.

TABLE III

Embryo development and time after fertilization.	Degeneration phenomena.	Development of embryo-sac and neighbouring tissues.
Early segmentation (0-3 days)	Degeneration of synergidae	Multiplication of antipodal cells.
Undifferentiated growth (3-6 days)	Degeneration of antipodals	Multiplication of endosperm nuclei. Embryo-sac passes through first stage of extension. (Fig. z, c, d.)
(9 days)		Growth of nucellar pillar and vascular supply of carpel wall. Second phase of extension of embryo-sac begins. (9 days.)
(9-10 days)	Nucellar pillar begins to degenerate	Aleurone layer appears. (10 days.)
First appearance of stem and root apices, and coleoptile primordium. (11-17 days)	Degeneration of nucellar pillar and of endosperm in neighbourhood of stem apex, first leaf primordium, and scutellary meristem, and of coleorhiza at root apices.	Accumulation of starch in endosperm and protein in aleurone layer.
Appearance of first leaf primordium. (17 days)		
Appearance of further leaf primordia and secondary roots	Absorption of endosperm in neighbourhood of scutellum.	Desiccation of grain and completion of second phase of embryo-sac extension. (21-40 days.)

Thus immediately after fertilization the synergidae degenerate, and at the same time the development of the embryo begins. During the degeneration of the antipodals the free endosperm nuclei increase in number, notably in the neighbourhood of the antipodals; the nucellus also undergoes an extension growth which is related to the disappearing antipodals. Local endosperm degenerations are associated with the development of the stem apex, the scutellary meristem, and the first leaf primordium, and radicle development also with the degeneration of the coleorhiza in its neighbourhood. Differentiation of the aleurone layer occurs during the absorption of the nucellar pillar, and in a similar way, the dissolution of the endosperm by secretions of the epithelium of the scutellum is correlated with the further growth of the embryo.

The correlations of these processes may be related to the nutritional value of the resorbed tissues, or to the liberation of growth-promoting substances which stimulate the development of cells nearby. There is no doubt but that these degenerating cells supply nutrients, for with the exception of the

depleted layers of the endosperm and of the nucellar pillar they are completely absorbed. These two tissues differ from the others in being of considerable extent, and are likely, therefore, to be primarily nutritional, nor are they associated with an essentially local development of a neighbouring part, but rather with the general growth of the embryo-sac. It is towards the earlier degenerations that attention may be specially directed as sources of nekro-hormones and other growth-promoting substances.

Before examining these phenomena in greater detail it is necessary to review the considerable literature on the significance of the synergidae, the function of the antipodals, and the work of Haberlandt and others on the formation of nekrohormones.

The prominent development of antipodals in the Gramineae, Ranunculaceae, Compositae, Scrophulariaceae, &c., has occasioned many speculations as to their possible functions. In these a direct or indirect nutritional role is usually emphasized. Thus Hofmeister (1849), who first described the antipodal complexes of angiosperms, believed that they provided for the nourishment of the endosperm as well as for that of the future embryo. Fischer (1880) figures in *Ehrharta panicea* degenerating antipodals with dividing endosperm nuclei in their neighbourhood, and remarks that it is probable 'that they have an important action in the filling in of the embryo-sac with endosperm tissue'. Westermaier (1890) describes the position and number of antipodals in many grasses and noted that the formation of endosperm nuclei is abundant in their vicinity. He also considers their function as nutritional. On the grounds of their elongation they are believed in some families, e.g. the Ranunculaceae, to be conducting, while in others they may have a synthetic function in the elaboration of food materials. Osterwalder (1898), who made a detailed description of the antipodal apparatus of *Aconitum Napellus*, also ascribes to them a nutritional function. The food materials reach the embryo-sac by way of the antipodals, which therefore assume a glandular function, and this author draws a comparison between the appearance of these cells and their nuclei, and those of other known glandular structure. Goebel (1905) suggests that the antipodals may secrete an enzyme which brings about the dissolution of the nucellus.

On the basis of their anatomy Lütscher (1905) separates antipodals into three types. In the first, which comprises those of naked nuclei, the function is believed to be concerned primarily with the solution and absorption of the nucellus. In the second, the antipodals form a differentiated cell complex which assists in the manufacture and transformation of food material for the embryo-sac. The third type includes only the haustorial antipodals of the Compositae, Rubiaceae, &c. Lütscher recognizes that various combinations of these types may exist, and considers that the antipodals of the Gramineae belong to the second type concerned with the manufacture and elaboration of food materials. This author also remarks that the development of large endosperms in all cultivated grasses may be connected with the size and

number of the antipodals. Huss (1906) gives an exhaustive review of the previous literature on this subject and conducts a number of microchemical tests to determine the function of the antipodals. This author reaches the conclusion that they play no physiological role in the development of the fruit, and regards them merely as the remnants of the female prothallus, which have a prominent development in some forms solely because they lie along the nutritional stream from the chalaza. The more recent works of Shadowsky (1926) and Jachimsky (1937) add nothing to the theories of previous authors. In addition to the works cited above numerous other authors have expressed similar views.

The possible role of nekrohormones may now be considered. The classical researches of Haberlandt (1913–23) on the production of nekrohormones by wounded plant tissues are too well known to require detailing, but his work dealing with the production of adventitious embryos is particularly important in this connexion. In the first of these later researches Haberlandt (1921a) describes the artificial production of parthenogenetic embryos in 'castrated fruits' of *Oenothera Lamarckiana* after local injury of the carpel; the embryo either develops from the unfertilized egg-cell or from the ruptured nucellus or integuments. It is suggested that development proceeds because of the liberation of wound hormones from the injured parts. These experiments have been called in question recently by Beth (1938) who was able to induce parthenocarpy by wounding, but did not succeed in bringing about the production of adventitious embryos. Beth concludes that in all probability Haberlandt's results can be attributed to incomplete castration. Haberlandt's figures of embryos arising from outgrowths of the nucellus furnish, however, definite evidence of his contention. In subsequent publications Haberlandt (1921b, 1921c) applies this theory to normal parthenogenesis, in which case the nekrohormones may be produced by the degenerating synergidae, the cytoplasm of the embryo-sac, or the antipodals. In normal fertilization also, he considers the possibility of a number of injuries which may liberate the necessary hormone, and which may act in minimal concentrations.

Orsós (1936) approaches the problem of the production of nekrohormones from a chemical standpoint; by means of the hydrolysis products of various proteins he simulated the action of the wound hormone. By addition of these substances to tissues growing under sterile conditions Orsós was able to obtain enzyme activation, cell division, and cell growth. Tyrosine by itself was found to stimulate growth, and the author suggests that the wound hormone may be chemically related to this substance.

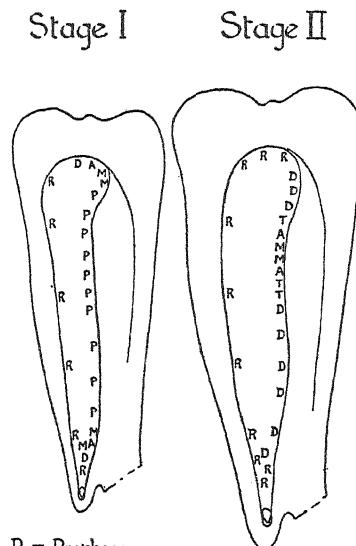
Recently Bonner et al. (1938) extracted a growth-promoting substance from injured bean pods. This was purified by various filtrations, extractions, and precipitations until an approximately pure, stable, and very active product was obtained. This substance 'traumatin' proved to be distinct from auxin, and was identified with the wound hormone of previous authors.

There is therefore sufficient evidence available as to the reality of nekro-

hormone (or wound hormones) profitably to consider their role in the development of the rye grain. Further evidence may be drawn from a recent paper by La Rue and Avery (1938) concerning the growth of the immature excised embryo of *Zizania aquatica* in artificial culture. These investigators found that embryos removed from the seed before the closing of the coleoptile pore, and cultured on agar containing nutrient salts, underwent increase in size but showed no evidence of further cell division. It has been shown above that in rye before closure of the coleoptile pore the stem apex and later the first leaf primordium protrude, and that in their neighbourhood degeneration of the immature endosperm cells occurs. This may possibly account for the arrested development of the excised embryos in *Zizania* if the conditions in this plant are similar to those found in rye; it may be attributed to the lack of the necessary hormone derived from the degenerating tissues. A further observation by La Rue and Avery may be cited in this connexion. In *Zizania* the scutellum is much elongated and probably grows, as in rye, by the activity of a terminal meristem. This growth in rye is associated with the degeneration of the endosperm, and the arrest of development of this meristem in excised immature embryos may be attributed to the removal of the influence of the endosperm tissue.

A closer examination of the degeneration of the antipodal and the behaviour of the neighbouring tissue reveals additional evidence for the suggestion that the former are concerned in the production of growth-promoting substances.

Of more than twenty preparations examined at the free nuclear stage of endosperm formation, two only showed dividing nuclei. Both these cases are represented diagrammatically in Text-fig. 5, I and II. In Pl. XXII, Fig. 2, the arrangement indicated diagrammatically as Stage II is shown in greater detail together with the appearance of the neighbouring tissues. In both, the stages of nuclear division found are not distributed at random among the nuclei: on the contrary, an orderly sequence is apparent. Starting from the region on the ventral wall nearest to the degenerating antipodal, and tracing the nuclei in either direction around the periphery of the embryo-sac a sequence of progressively later stages of mitosis is encountered. This



P = Prophase
M = Metaphase A = Anaphase
T = Telophase D = Reconstituted
R = Resting nuclei daughter nuclei

TEXT-FIG. 5. Nuclear divisions within the embryo-sac, showing the orderly arrangement of stages in mitosis.

appearance, seen in section, is found to hold also when the arrangement of nuclei in three dimension is reconstructed. Again, passing from a focus at the degenerating antipodals, the same orderly sequence holds in all directions.

In Stage I prophases occur along the greater part of the ventral wall of the embryo-sac succeeded by metaphase and then anaphase at the distal and micropylar ends, and resting nuclei along the dorsal wall. In Stage II, pictured in the diagram, the focus of the arrangement is marked very clearly by nuclei in metaphase. Above and below are nuclei in anaphase succeeded in both directions by telophase and then reconstituted daughter nuclei. Thus the phases of division in Stage II repeat at the focus of the arrangement the relations found in Stage I along the whole ventral side of the embryo-sac. The final remnants of the antipodal nuclei are most abundant opposite the nuclei in metaphase in Stage II, and therefore are likely to be concerned in this arrangement.

Such a distribution would result if, at the point represented by the degenerating antipodals, nuclear division is initiated, and as it proceeds the dividing nuclei move outward in every direction along the periphery of the embryo-sac and accomplish their division before the dorsal side of the latter is reached. Stage I, therefore, must precede Stage II and this is corroborated by the fact that the length of the embryo-sac is less in the former, which must therefore represent an earlier stage.

The alternative view that a wave of nuclear division originates at the focus and passes along the periphery is excluded, since the observed arrangement is incompatible with such a view.

The suggested explanation of the orderly arrangement observed involves an actual migration of nuclei during the progress of cell division. It is well known that the prophase stages are relatively long as compared with the later stages, so that during a phase of nuclear division the largest number of any stage encountered will be prophase. This, in fact, is the case in the conditions shown in Stage I.

The movement of the nuclei is away from the focus in every direction, so that there is no question here of the circulation of protoplasm, known to occur in mature cells, but rather a rapid synthesis of cytoplasm in the focal regions and an orbital flow. The rapid extension of the embryo-sac at this time, already referred to, presupposes a synthesis of protoplasm, since the lining layer of cytoplasm actually becomes thicker during extension growth, and this is particularly so in the region of maximum nuclear division. It may be presupposed, therefore, without doing violence to observed facts, that the nuclei are carried forward in this stream, with the result that later stages of mitosis always occur farther away from the focus of nuclear division. It further seems probable that in the actual region of synthesis some nuclei remain stationary and thus, by the movement postulated, the nuclei in the focal region are not depleted but on the contrary remain constant in number by multiplication.

The observed anatomical facts rather support the view suggested, since the vascular trace in the carpel wall terminates opposite the focal region and the cells of the nucellus, in so far as they have a translocatory function, are orientated in such a way as to deliver nutritive material for synthesis precisely in the requisite region. The extension growth of the nucellar cells which would considerably aid this translocation may therefore be regarded as subservient to this organizing activity in the circumscribed region of the embryo-sac.

It has already been remarked that both Fischer (1880) and Westermaier (1890) noted the division of endosperm nuclei in the neighbourhood of the antipodals. The figure given by Fischer does not indicate any regular arrangement of the phases of division in the dividing nuclei. Osterwalder, however, includes a figure of the embryo-sac of *Aconitum Napellus* (after Dürer) in which a somewhat similar arrangement of the dividing endosperm nuclei to the one here described is shown, though not so clearly. At the micropylar end of the embryo-sac are drawn only telophase and anaphase, and at the chalazal end only metaphase. This author apparently overlooked the regularity of the arrangement.

The conclusion may be drawn from this evidence that the antipodals actively stimulate nuclear division in the endosperm. As to the nature of this stimulation there is no direct evidence. As Snow (1935) has shown, activation of the cambium of the higher plants can be induced by auxin, and this relation has been further emphasized by Söding (1936) in the secondary growth of trees. The well established effect of auxin on extension growth would appear to be also concerned in the effects of the antipodals. The whole arrangement of cells 'radiating' from the position of the degenerating antipodals and resembling a diffusion field, strongly suggests that the antipodal nuclei are also concerned in the production of substances promoting extension growth. The very local nature of the extension growth of the nucellus during the phase of the degeneration of the antipodals lends some support to this view. Meanwhile the embryo-sac is passing through the first phase of rapid extension, so that we must assume that the auxin produced affects, to a lesser degree, all the tissues surrounding the embryo-sac. Until about three days after fertilization there is very little expansion of the embryo-sac; during this time the antipodal nuclei are increasing in number. Whereas the increase in length of the embryo-sac is only 13 per cent. per day during this period, during the next two days the increase averages 113 per cent. Simultaneously with this increase the antipodals degenerate, and they disappear by about the sixth day. The complete disappearance of the antipodals is followed immediately by almost complete cessation in the growth of the embryo-sac, and it is not until about the ninth day that active growth is resumed.

The main change, occurring subsequently, external to the embryo-sac, is the rapid nuclear divisions in the nucellar tissue leading to the production of the nucellar pillar. The differentiation of the vascular strand in the

neighbouring region of the carpel wall may indicate that growth-promoting substances responsible for this development are translocated from the dying regions of the plant below, for at this stage rapid death of the leaves of the plant is occurring. Further evidence on this point arises from the effect of the removal of ears during this phase. Under these conditions mature embryos are obtained, but these are dwarfed, and the extension of the embryo-sac is arrested to a greater or less extent depending on the time of removal of the ear from the plant. These dwarf embryos will be described in a later communication.

In conclusion the author has much pleasure in recording his thanks to various members of the staff of the Botany Department of the Imperial College. To Professor F. G. Gregory the author is indebted for suggesting the subject of research and for advice and criticism during its prosecution. The interpretations of the results have profited much from his suggestions. To Dr. O. N. Purvis the author is grateful for permission to use dated material from her vernalisation experiments, and for much timely assistance during the course of the work. On matters of cytological technique the author has been guided by Mr. F. Howarth, to whom also the interpretation of the observed orderly arrangement of nuclear divisions in the endosperm is due.

SUMMARY

1. The development of the embryo and fruit of rye has been reinvestigated, and is shown to be in the main similar to that of other cereals. In the following respects the results recorded extend previous observations.

2. The origin and developmental changes leading to the giant antipodal nuclei previously observed are recorded in detail; the characteristic large masses of chromatin are shown to be the condensed reticula of the seven haploid chromosomes.

3. An important post-fertilization development of the nucellus is described in detail. During the extension of the embryo-sac the nucellus at the ventral groove undergoes a new development to form a pillar of meristematic tissue (the 'nucellar pillar') which traverses the entire length of the caryopsis and is associated with the further differentiation of the vascular trace of the raphe (Text-fig. 2). The initiation of the aleurone layer is accompanied by almost complete digestion of the nucellar pillar in its neighbourhood (Pl. XXII, Fig. 1).

Quantitative data on the growth of the embryo and embryo-sac are presented, and the correlative changes in growth-rate and progress of differentiation in the fruit are stressed. The chief results obtained are as follows.

4. Until twenty-two days after fertilization the relative growth-rate of the embryo remains constant; cell divisions occurring throughout the embryo once each day during this period. Cessation of exponential growth coincides with the beginning of the desiccation of the fruit (Text-fig. 3).

5. The growth of the embryo-sac is discontinuous, consisting of three distinct phases (Text-fig. 5). There is (i) an initial increasing rate of growth to about six days, (ii) cessation of growth for about three days, and (iii) resumed rapid growth, with a decreasing rate to maturity. The most rapid growth during the first phase is correlated with the degeneration of the antipodals, while the segmentation of the endosperm occurs during the second phase. The initial extension of the embryo-sac is more rapid in spring than in winter rye, though the final grain size is the same in both varieties.

6. A series of degenerative changes is described associated with each phase of development, five such being distinguished: (i) synergidae, (ii) antipodals, (iii) immature aleurone layer in neighbourhood of embryonic primordia, (iv) nucellar pillar, (v) endosperm in contact with scutellum. The relation of these to further developments is schematized in Table III, and it is suggested that hormones are liberated from the degenerating tissues. In support of this hypothesis the following additional evidence is presented.

7. An exact correlation exists between the first phase of embryo-sac extension and the disappearance of the antipodal nuclei (Text-fig. 5 and Table II).

8. The anatomy of the nucellar pillar (Text-fig. 2 and Pl. XXII, Fig. 2) may also indicate the diffusion of an extension growth-promoting substance from the neighbourhood of the degenerating antipodals.

9. A regular arrangement of the phases of mitosis of the free endosperm nuclei within the embryo-sac (Text-fig. 6 and Pl. XXII, Fig. 2), having a focus in the region of the degenerating antipodals is described. It is suggested that nuclear division is initiated at the focus, possibly by a hormone, and that the dividing nuclei are carried outwards in an orbital flow of cytoplasm.

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EXPLANATION OF PLATES XXII AND XXIII

Illustrating Mr. Nutman's article on 'Studies in Vernalisation of Cereals. VI. The Anatomical and Cytological Evidence for the Formation of Growth-Promoting Substances in the Developing Grain of Rye'.

PLATE XXII

Fig. 1. Median longitudinal section of grain showing the dissolution of the nucellar pillar in the neighbourhood of the aleurone layer. *al.*, aleurone layer: *en.*, starch storage parenchyme of endosperm: *n.*, nucellar pillar: *v.b.*, vascular bundle of raphe: *d.*, depleted nucellar tissue.

Fig. 2. The division of the endosperm nuclei on the ventral wall of the embryo-sac as seen in the median longitudinal section of grain. The extension growth of the nucellar pillar is also shown. The section is slightly oblique so as not to include the vascular bundle of the raphe: instead, here the nucellar pillar is bounded by the epidermis of the nucellus. References to tissues as above, and in addition: *A.*, remnants of antipodal nuclei: *epi.n.*, epidermis of the nucellus: *es.*, vacuole of embryo-sac.

PLATE XXIII

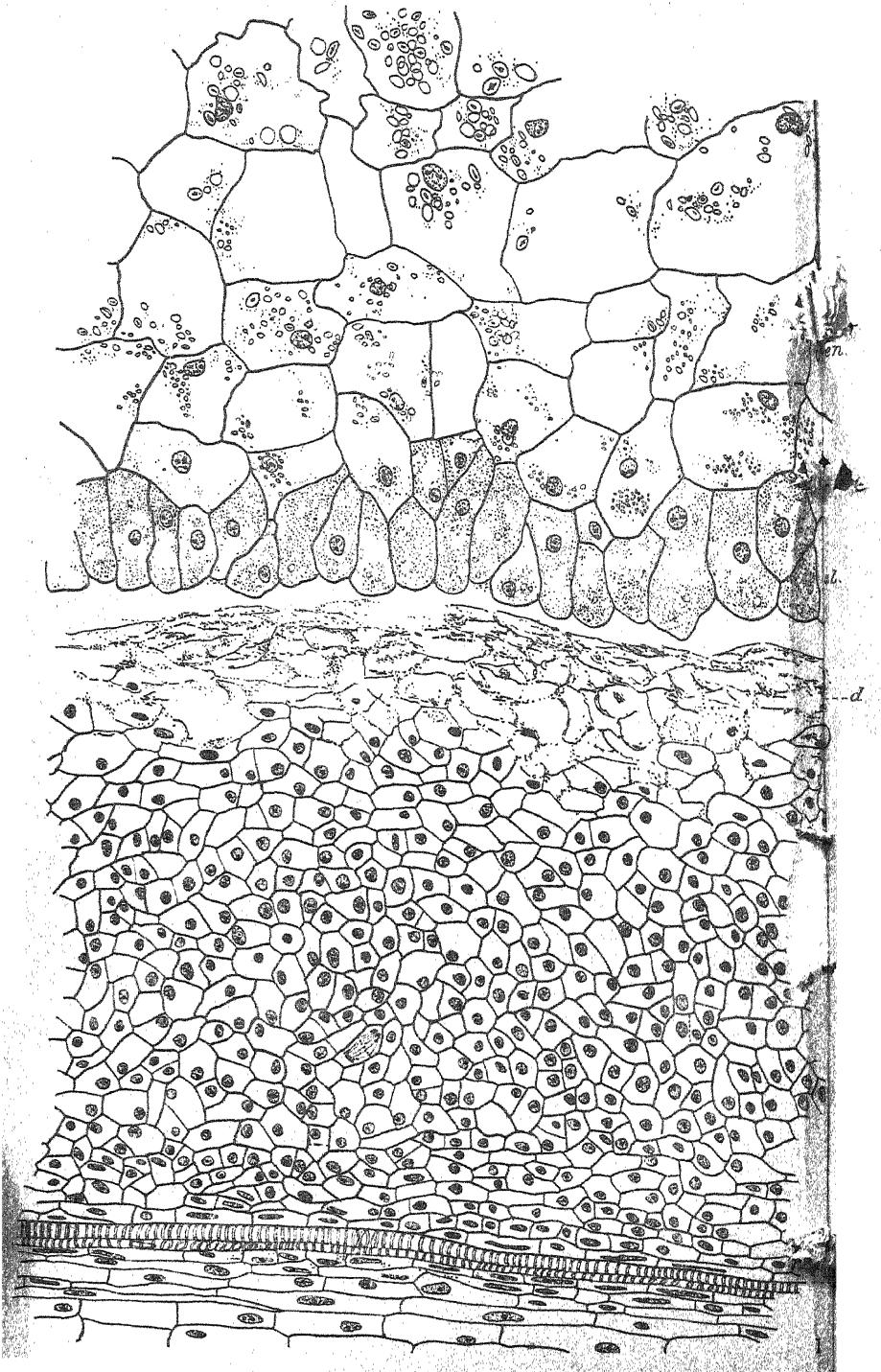
Growth and degeneration of antipodal nuclei. All figures drawn to the same scale.

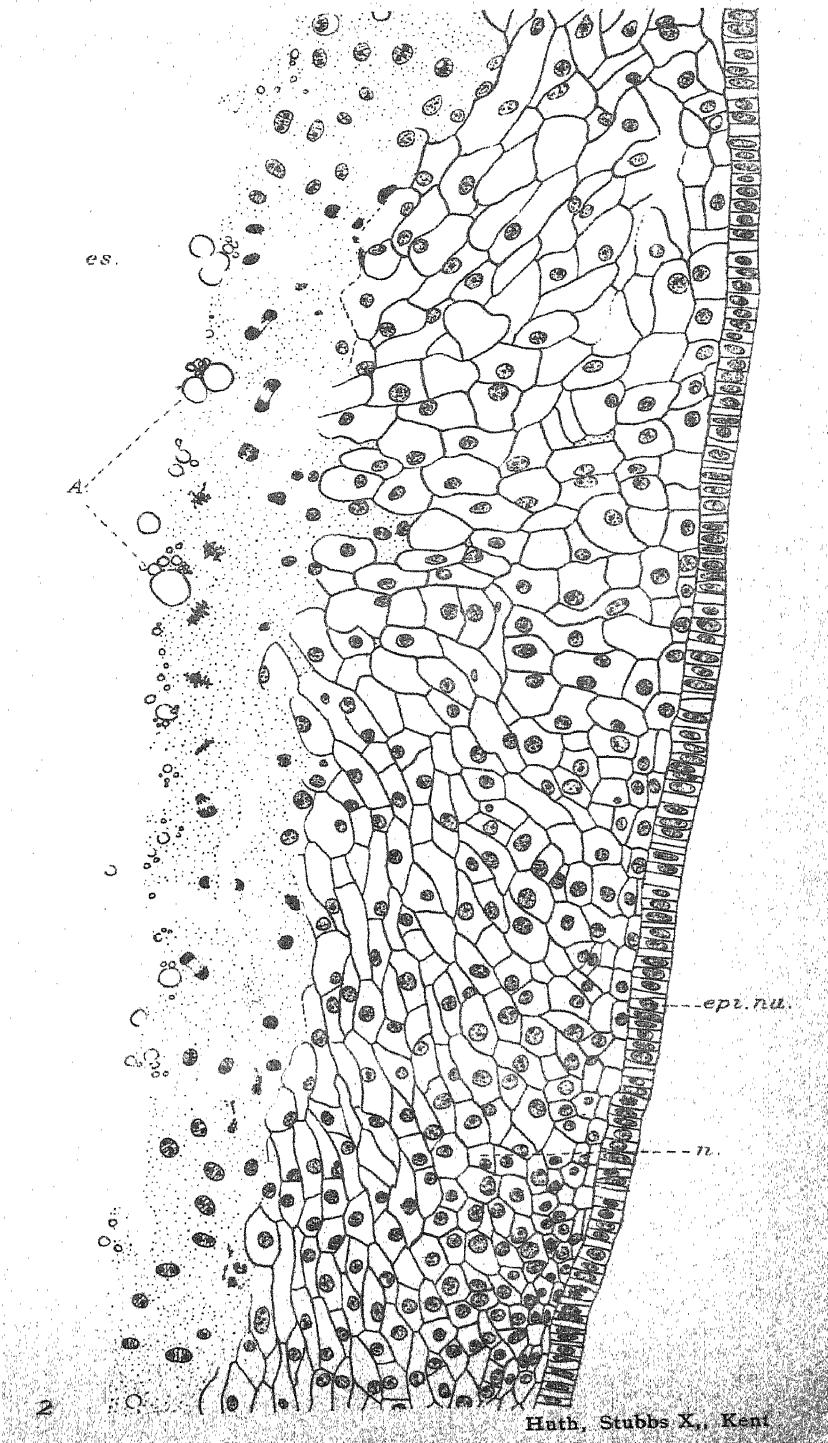
Fig. 3. Transverse section of the embryo-sac before fertilization, showing three antipodal nuclei.

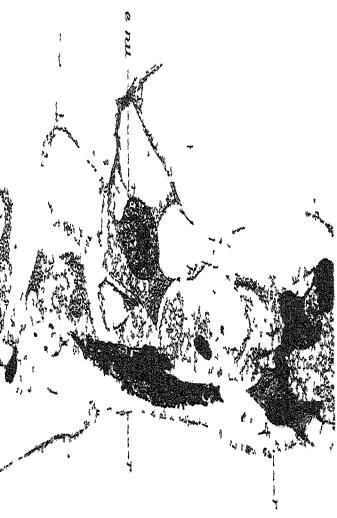
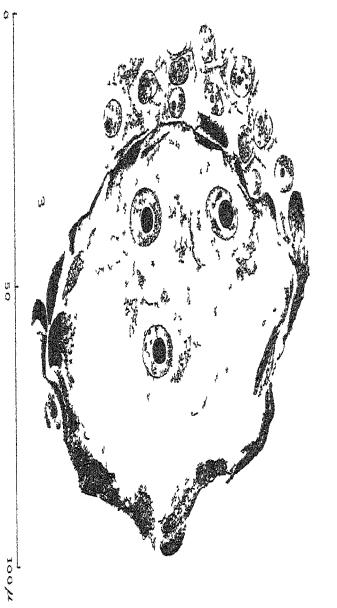
Fig. 4. An antipodal nucleus at about the time of fertilization of the ovum.

Fig. 5. Three antipodal nuclei five days after fertilization (winter rye) to show the arrangement of the chromatin and beginning of the breakdown of the nuclear membrane.

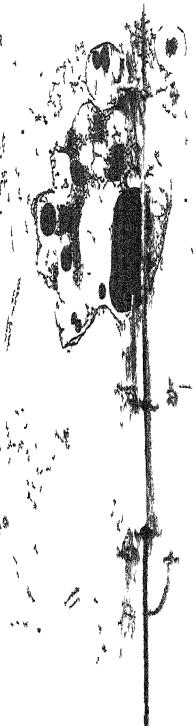
Fig. 6. Further stages in the degeneration of the antipodal nuclei. Remnants of possibly three nuclei are indicated (*r*) and a fourth (*a*) with the nuclear wall still intact: *e.nu.*, endosperm nucleus.







0
50
100 μ



6



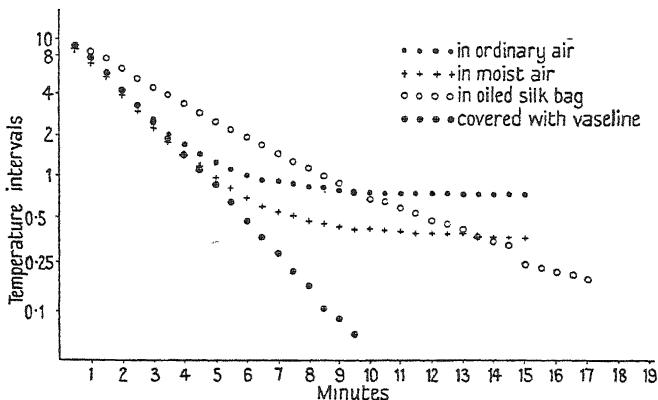
nucleus

NOTES

THE RATE OF TEMPERATURE CHANGE WITHIN THE PLANT.

—The authors, while engaged on work which involved keeping plant material at known temperatures, were unable to find in the literature applicable data on the rate at which such material attains the temperature of its surroundings. Direct determinations were therefore made, which indicate the necessity for caution in the apparently simple technique of bringing plant material to a known temperature.

Two thermostats were used. One, intended for field work, consisted of an aluminium cylinder 9 cm. in diameter and 25 cm. in length, with closed ends, heated by



resistance wire wound on its outside. The cylinder itself acted as an expanding element and operated a contact in conjunction with an Invar rod. Air inside was not stirred, and regulation was of the order of $+0.5^{\circ}\text{C}$.; the 'hunting' period being about 1 minute. The other thermostat was larger and more accurate. It contained 60 litres of air blown in a confined circulation, passing over a grid of heating wires and through a special thermo-regulator; the whole chamber was lagged with a layer of sawdust 7 cm. thick. A sensitive thermometer showed no fluctuations as great as 0.1°C . over a long period of time. The 'hunting' cycle was 17 seconds.

Determinations were made by copper-constantan thermo-couples, the junctions being of 42 S.W.G., soldered and protected by a very small fold of waxed paper. The mirror galvanometer (kindly lent by The Royal Society) was of 52 ohms, 8 seconds period, wired to give a scale deflection of 4.5 cm. per degree centigrade and critical damping. Rapid switching of the couples to the galvanometer was effected by mercury contacts.

The material was: young inflorescences of *Tradescantia bracteata*, *Secale cereale*, and bulbs of *Tulipa Hageri* and the commercial tulip 'Farncombe Sanders'.

A typical experiment, chosen because the same inflorescence was used for the several conditions, is represented on the graph. This *Tradescantia* inflorescence was

first allowed to attain room temperature (14.8° C.) and then suddenly transferred to a temperature of 24.1° C. in the large thermostat. Readings were taken every 30 seconds. The rate of heating of the same inflorescence over the same temperature interval was observed under the following four conditions: (a) in ordinary air, (b) after an attempt to saturate the air, (c) with the inflorescence enclosed in a small oiled silk bag, (d) after the inflorescence had been dipped in melted vaseline.

In ordinary air, evaporation is responsible for preventing the material from attaining a temperature nearer than 0.8° C. of the thermostat. This inflorescence had only one bract; larger inflorescences, with leaves, reach equilibrium at 2° or even 3° C. from the surrounding temperature. Attempts to saturate the air in order to stop evaporation were ineffective. In the most successful of these attempts, a soaked towel (2 ft. \times 3 ft.) was fully exposed to the air current, and here equilibrium was reached at 0.4° C. less than the temperature of the thermostat. The failures of attempts to saturate the air are probably due, in part at least, to the rapid fluctuations in temperature which are not detectable by a mercury thermometer, or other instrument of appreciable heat capacity. Thus, in the large thermostat, a bare junction showed a fluctuation of 0.5° C. within each 'hunting' period of 17 seconds, whereas no fluctuation could be detected after the junction had been embedded in a piece of paraffin wax the size of a pea.

Two methods overcome the evaporation effect: dipping the inflorescence in melted vaseline, and enclosing it in an oiled silk bag. The second method was adopted as the practical solution. Provided the oiled silk bag was as small as possible and moistened inside, it was found that even the largest inflorescence was within 0.1° C. of the temperature of the surrounding air current in 18 minutes. It should be noted that the bag used in the experiment illustrated did not fulfil the above conditions.

Rates of heating of other material are given in the table below. The second column gives the difference between the room temperature (which was that of the material when placed in the thermostat) and the thermostat temperature. The third column gives the difference of temperature between the material and the thermostat when the material had reached a steady temperature. The last column gives the time taken to reach that steady temperature.

Material.	Temperature difference between		
	room and thermostat.	material and thermostat.	Time taken.
<i>Secale cereale</i> with bag	10.4° C.	0.07° C.	14 minutes
" without bag	10.4° C.	1.9° C.	30 "
<i>Tulipa Hageri</i>	9.8° C.	0.4° C.	52 "
Farncombe Sanders	12.0° C.	0.6° C.	66 "

The data given in this note will, it is hoped, warn experimenters that the process of bringing plant material to a known temperature may require care.

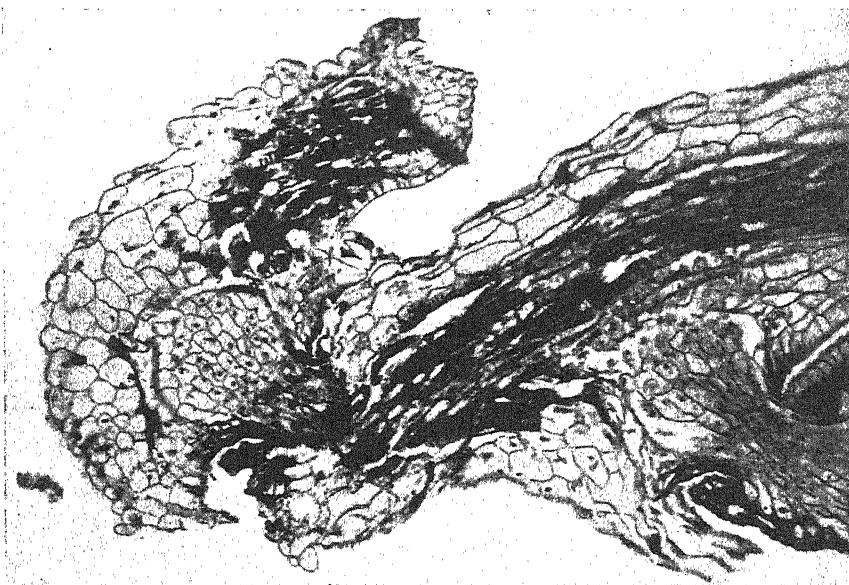
C. J. CULLEN.

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LONDON.

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SELF-PARASITISM IN CUSCUTA.—During the course of preparation of a set of microtome sections of *Cuscuta epithymum* Murr. upon *Calluna vulgaris* Hull. as host an interesting example of self-parasitism was observed. Sections through one



of the specimens showed that in one region, where two parasite stems had come into contact, the outermost thread had developed a haustorium which had penetrated the cortex of the innermost thread and had applied itself to the vascular system. This is clearly visible on the left of the photograph. In the right-hand bottom corner a haustorium from the innermost thread is seen filling a leaf of the host plant. It would be interesting to know how frequently self-parasitism of this type occurs.

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The Conception of the Satellite and the Nucleolus, and the Behaviour of these Bodies in Cell Division¹

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With one hundred and thirty-eight Figures in the Text

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I. INTRODUCTION

THE nucleolus has been the subject of numerous investigations ranging over the last century and a half. Its behaviour in cell division, its organization and composition, have all been studied. However, apart from recent light on its origin and composition, the problem is still shrouded in speculations regarding its function. These varied views are to be found enumerated in Ludford (1922), Wilson (1925), and Sharp (1934). It therefore seemed necessary to study the nucleolar behaviour and establish some of the conclusions on a sound basis before venturing on interpretation of its function. Excepting the bare fact that the nucleolus appears in telophase and disappears in the succeeding prophase, in general, in all organisms, there is nothing very much understood. We have no full understanding of its behaviour, organization, or composition. The brilliant work of Heitz (1931 *a*, *b*) and McClintock (1934) has cleared much of the mystery of the nucleolar organization. Some work has also been done on its composition. The present paper deals with the behaviour of the nucleolus, in general, and its relation

¹ Part II of thesis approved for the Degree of Doctor of Philosophy in the University of London.

to the chromosomes in particular. It is attempted to deduce thereby the part it plays during cell division.

The work was carried out in the Botany Department, King's College, London, under Professor R. Ruggles Gates.

The present investigation firmly establishes a close association between the nucleolus and the locus of its organization, strongly suggesting interchange of material between the nucleolus and the chromosomes. Also, the nature of the satellite is studied in relation to the nucleolus and a new interpretation suggested.

Material and methods.

Seventeen species of Allium, including 11 diploids, 4 tetraploids, and 2 hexaploids, were examined. They are as under:

Diploids: *A. Cepa* L., *A. sativum* Linn., *A. ciliicum* Boiss., *A. siculum* Ucria, *A. Sewerzowii* Regel, *A. Scorzoneraefolium* (DC. in) Red., *A. Wallianum* Steud., *A. nigrum* L., *A. darwasicum* Regel, *A. decipiens* Fisch. Hort., and *A. amplectens* Torr.

Tetraploids: *A. Bidwelliae* S. Wats., *A. cyaneum* Regel, *A. Déséglisei* Bor., and *A. margaritaceum* Sibth. & Sm.

Hexaploids: *A. giganteum* Regel and *A. senescens* L.

The bulbs were kindly supplied variously by the Chelsea Physic Garden and by the Royal Botanic Gardens, Kew and Edinburgh. For root-tips they were grown in the greenhouse in half-and-half mixture of sand and soil. For meiotic study the pollen mother-cells were fixed on the spot whenever possible.

The following were the fixatives used: (1) Navashin (Karpechenko), (2) 2BE, 2BD (La Cour), (3) Benda. Navashin proved to be the best for nucleolar study in meiosis as it almost always leaves the nucleolus unstained while the chromosomes are stained purplish in gentian violet.

Technique.

For meiotic studies, the usual gentian violet technique was invariably applied. For the nucleolar study in mitosis Altmann's aniline-fuchsin-picric-acid method (McClung, 1929) and Feulgen root-tip smear counterstained with orange G were successfully employed. The Feulgen smear technique has been described elsewhere (Mensinkai, 1939a). Only the counterstain application will be given here.

After the smeared slides are taken up to clove oil, a drop or two of orange G prepared after Chamberlain (1932) is left on them; they are then laid horizontal for a few minutes, all the time observing the stain under a differentiating microscope. When the nucleus is just yellowish the slides are put back in clove oil or fresh oil is added by a drop-bottle on the slide which is immediately taken to xylene for mounting. Root-tips were sectioned from 12–16 μ and flower buds not less than 25 μ , as thinner sections were found to cut the

nucleus. All drawings are made at bench level with the aid of Reichert camera lucida, using a 2 mm. apochromatic objective N.A. 1·4, an aplanatic condenser N.A. 1·4, and an ocular K18, $\times 20$, giving a magnification of approximately $\times 2900$. Figs. 1-4, 36, 52, 60 magnification ($\times 3400$). Cell-wall is omitted in all telophase drawings except Fig. 62.

Observations.

A careful study was made of the behaviour of the nucleolus in relation to the chromosomes both in mitosis and meiosis as well as in relation to the chromosome sets in diploids, tetraploids, and hexaploids. The nature of the satellite and its relation to the nucleolus has also been discussed.

II. THE NATURE OF THE SATELLITE

Preliminary statement.

Mitosis, as has been shown (Mensinkai, 1939a) is a spiralization cycle of the chromosomes, which become shortest and thickest in metaphase and longest and thinnest in interphase. In interphase, the chromosome is released of most of its minor spirals and attains its maximum extension. Presumably no satellite is present at this stage. Being a rolled end of the chromonemata it unrolls like the rest of them. It is difficult to be definite about this, as despite the maximum thinness of the interphase thread, despiralization is never complete until the following metaphase. However, being the terminal portion, the satellite might be assumed unrolled. The SAT-filament contains chromatin and is a spiral as well as a continuation of the chromosome chromonemata, and the satellite itself remains in firm association with the nucleolus at the point of its organization.

With the onset of prophase, spiralization commences and the elongated chromonemata shorten and tend to form the condensed metaphase chromosomes by developing new spirals. The SAT-filament is hindered in this contraction (by spiralization) due to its contact with the surface of the nucleolus. The satellite segment of the chromonemata, however, does spiralize. It remains as a satellite and is characteristically constant for a tissue of a species. As prophase advances, the chromosomes shorten while the SAT-filament remains extended as in the resting stage. By the beginning of metaphase the nucleolus will have generally disappeared and the shortening of the chromosome is maximum. In metaphase the filament, along with the satellite, is visible, presumably because it cannot spiralize owing to the nucleolar disappearing so late that its proteins have approached the isoelectric point and consequently the chromosomes cannot spiralize further. From metaphase to anaphase (*tassement polaire*) there is little or no contraction and so the filament remains as such.

In telophase and interphase the chromosomes elongate, the nucleolus remaining in contact at the position of its organization.

(a) *The satellite.*

The conception of the satellite or trabant arose with its discovery by Navashin (1912) as a body attached to the nucleolus in the resting stage. He supposed it to be removed by the condensing chromosomes later in prophase; similar attachments have since been recorded by several workers, but the conception of the satellite and its filament even at present is vague. Heitz (1931b) does not state how the filament arises although he describes the nucleolus as developing round about the filament, while McClintock (1934) makes a bare mention that the filament arises secondarily as the nucleolus is organized, presumably due to the growth of the latter. Up till 1937 the satellite was regarded as a chromatic knob with an achromatic stalk or filament, while, as described in most current papers, the term satellite implies the stalk and the knob or head as its composite parts. Further, Heitz (1931b) describes the filament as achromatic and devoid of thymo-nucleic acid, that is, not giving Feulgen nucleal reaction. Resende (1937b) remarks that the filament either lacks thymo-nucleic acid or is poor in it. Fernandes (1937) finds in Narcissus that the SAT-filament does give Feulgen reaction. This paper came to my hands in March 1938, and the chromaticity of the filament was independently observed by me, in December 1937, in *Allium ascalonicum* (*shallot*), after applying the Feulgen test to a root-tip smear of the species. The filament is not only chromatic, containing nucleic acid, but is also a continuation of the chromonemata for the following reasons:

- (i) Like the chromonemata, the satellite, as well as the SAT-stalk, gives Feulgen reaction (Mensinkai, 1939a).
- (ii) As is popularly understood, the satellite with the filament is an appendage to the chromosome.
- (iii) The SAT-filament generally corresponds in thickness with the chromosome thread in the resting stage, although at times it is thinner. Hence the satellite and the filament suggest themselves as nothing more than parts of this chromosome spiral.
- (iv) The thickness of the SAT-filament corresponds to that of the chromosome thread seen in a constriction connecting the adjacent segments of a chromosome. The constriction can no longer be regarded as a gap between the segments of a chromosome arm. It is simply a continuation of the chromosome thread which either has no spiral or does not possess a spiral of the order of the rest of the chromosome; and it usually represents reversal of direction of the minor spiral. This is observed perfectly clearly in Fig. 3c (Mensinkai, 1939a), which is a root-tip smear of shallot in Feulgen reaction. The constriction of the chromosome is stretched out under pressure, while smearing. It seems clear that the chromonemata of the constriction are a continuation of the chromosome helix and, like it, are chromatic, giving

Feulgen reaction. In other preparations as well, in the root-tip of *Allium senescens* stained in gentian violet, similar constriction gaps have been seen bridged by the chromosome thread.

(b) *The SAT-filament is a spiral.*

Further, the SAT-filament seems to have a spiral of an order lower than the minor spiral possessed by the chromosome. Being a continuation of the chromonemata which shorten by spiralization, it is possible that this represents the simplest visible spiral of the chromosome before it takes on the minor spiral. Fig. 6a shows this spiral, while Figs. 6b, 7, 8, 15, and 16 represent the minor spiral. They are from *A. margaritaceum* root-tip preparations, sectioned 14 μ and stained in gentian violet.

(c) *The satellite, the rolled end of the chromonemata.*

Once the SAT-filament is recognized as a continuation of the chromonemata, the satellite obviously means the end segment of the filament. The only point that calls for explanation is why it is always knobbed. Since shortening and elongation of the chromosome is due to spiralization and de-spiralization, this swelling on the end of the SAT-filament appears to be a shortened or spiralized end of the chromonemata, fixation of which brings about fusion of the gyre or gyres to a solid homogeneous mass—the satellite.

Once formed, the satellite seems to persist from division to division, the nucleolar size being constant for the tissues of a species. The nucleoli vary from tissue to tissue, e.g. the pith nuclei were always observed to have larger nucleoli than the apical meristem nuclei of the root-tip in the same species. The chromosome size, as also the satellite size, seems to vary correspondingly.

The other line of evidence is that in the same species and in the same tissue the satellite is sometimes apparently missing and the filament comparatively longer. In some species where the nucleoli persisted to metaphase in somatic mitosis and the satellite remained attached to the nucleolus, the length of the filament was not constant. The same state of affairs was observable in the meiotic prophase.

A bulb species of *A. margaritaceum* with $2n = 32$ (Mensinkai, 1939c) is a tetraploid with two pairs of nucleolar organizing chromosomes, a pair of SAT-chromosomes and a pair of chromosomes with secondary subterminal constrictions. All four nucleolar chromosomes were never seen in one plate. Only one pair of satellites could be detected in a plate (Fig. 1). The other pair is inferred from their attachment to the nucleolus persisting to metaphase. These chromosomes are different in their morphology from the SAT-chromosomes. Fig. 4A shows such a chromosome. It is attached to the nucleolus by the long arm, presumably at a subterminal constriction, and cannot be mistaken for the SAT-chromosomes (Fig. 1). The normal satellite (Fig. 1) is very stout, with a very short filament situated on the short arm of the chromosome, and is of the S₁ type typical of *Allium*.

In Fig. 3b the nucleolus persists to metaphase and the chromosome is attached to it by a long, thin filament with practically no satellite. The homologous chromosome is likewise without any satellite (Fig. 3a). Fig. 2 is another instance of a chromosome with no satellite but only a fine filament.

In the seed species of *A. margaritaceum*, which proved to be a diploid with $2n = 16$ (Mensinkai, 1939c), the satellite chromosomes are similarly suggestive. In one anaphase, Fig. 5, the satellites are the normal S_1 type with equal filaments and satellites. Figs. 6b–9 are from metaphase plates and show only a thick filament. Fig. 10 is also a pair of SAT-chromosomes from metaphase. One of them shows the sister SAT-filaments separate, though the sister chromatids themselves are not visibly double. Of these two filaments, only one possesses a satellite. The other is a uniformly stout thread. Figs. 11–17 are from anaphase, none of them showing the satellite. In Fig. 11 the chromosome marked 'x' apparently seems to possess a satellite, but on careful focusing the 'satellite' turns out to be only the end of the filament curled up. Loss of the satellite is an inadequate suggestion. At least in this species the satellite appears definitely to be the rolled end of the chromosome.

Another species, *A. Sewertzowii*, affords equally significant evidence. It has a pair of S_1 type satellites which may be seen in several figures in diakinesis (Figs. 95, 96, 97, 98, 99, 104). Figs. 100 and 101 show no satellites, but fine filaments instead, which are presumably a combination of the filaments of both the homologous chromosomes of the bivalent. Fig. 96 (diakinesis) shows comparatively small satellites, and Fig. 97 (diakinesis) two long threads apparently under tension with two smaller satellites. Conclusive evidence could be had from Figs. 88, 89, and 90 representing diplotene, where only a long thread is visible in place of a satellite. In Fig. 90 the thread is masked by the overlapping bivalent. The species being a diploid with only a pair of satellites, these variations strongly support the view in question.

A. Cepa is still another species, the Egyptian variety of which offers a good deal of evidence for analysis in this respect. It is a secondarily balanced diploid with $2n = 16$ and two pairs of nucleolar chromosomes (Mensinkai, 1939c). One pair is the normal S_1 type SAT-chromosomes with a slight difference in the size of the satellites, the other pair are the chromosomes with secondary subterminal constrictions (Fig. 18).

Starting with this normal pair of satellites, Fig. 33, showing anaphase, can only be explained on the basis of unravelling of the satellite, as the filament is relatively double in length without any satellite knob. Also, never more than two satellites, nor more than two secondary constriction-chromosomes, form nucleoli in this variety.

Fig. 30 is from side-view metaphase. One chromosome has a scarcely visible thread. The other, its homologue, has the thread with four granules in descending order, the terminal one being the biggest. Fig. 20 is from side-view metaphase, with a pair of SAT-chromosomes attached to a common nucleolus. Owing to the firmness of attachment the filaments are stretched

and the satellites have fused to a single mass which is much smaller than the normal satellites.

Similarly, Fig. 22 is a side-view metaphase, showing long fine filaments and smaller satellites compared with the normal and attached to two separate nucleoli. In these two instances, it appears, the satellite has undergone slight unravelling, while the filament is drawn out from the chromosome as it spiraled and shortened considerably from interphase to metaphase. The nucleolus has apparently lagged behind in this movement, its greater viscosity offering resistance against the karyolymph as the chromosome shortened. The same is illustrated in Figs. 21, 23, 24–6. In Figs. 24 and 26 the satellite is considerably bigger than normal. This appears due to the attachment of the satellite to the nucleolus along with the filament which is possibly rolled up with the satellite and swollen in fixation. The filament in the figures is the drawn-out chromosome helix.

The granules on the filament in Figs. 23, 27–31 appear to be twists of the spiraled filament and not chromomeres brought in view because of the extreme extension of the chromosome, as at lepto-pachytene stage. In that case, wherever there is no satellite the 'chromomeres' should look more prominent, the filament then being in full extension. Also, they are not constant in size like the chromomeres and some of them are distinctly bigger than the chromomeres observed in pachytene in the same species. Fixation and fixatives seem to play a prominent part in causing this appearance. The gyres in close contact fuse when fixed and look like a single mass. Then fixatives also leave the chromosomes swollen to different degrees. Navashin's fluid, for instance, always swells the chromosomes much more than any of the Flemming modifications. Sometimes differential swelling seems to be due to differential reactivity of the chromosomes to the fixative. Fig. 23 shows the tandem satellite as observed by Taylor (1925) in one plant of *A. Cepa*. The suggestion of translocation to account for the tandem condition does not, however, apply here, since the other satellite also is present on the homologous chromosome.

Fernandes (1936) describes similar variations in the satellite size and the length of the filament, from no satellite to no filament with many intermediate stages. On the whole the variations in the size and shape of the satellite and the filament do not seem unintelligible on the hypothesis that the filament undergoes changes in length and shape due to attachment of the satellite to the nucleolus. In the light of these facts, Navashin's (1926) results, distinguishing three types of plants in *Crepis* with two, one, or no satellite in the ratios 42 : 90 : 42 require reinterpretation. These interpretations about the nature of the satellite appear to explain some of the incongruities observed in meiosis.

(1) It is a matter of frequent occurrence and observation that the satellite observable in somatic metaphase is not at all to be seen in the first metaphase of meiosis. The chromosomes in meiotic metaphase are always observed to

be shorter than the somatic metaphase chromosomes, indicating greater spiralization. From the above viewpoint about the satellite as a continuation of the chromonemata themselves, it is likely that, due to this greater spiralization of the chromosomes, the filament, along with the satellite, gets included within the bivalent chromosomes. Another important factor is that the nucleolus is never seen persisting until first metaphase, so that the SAT-filaments will have a chance to spiralize unobstructed by the nucleolar presence and get merged into the chromonemata of the bivalent chromosomes.

(2) The satellite in meiotic prophase sometimes appears bigger than at somatic prophase in the same species. (Figs. 18, 79 and 83.) This is explicable on three grounds. First the satellite in meiotic prophase is a fusion of a pair of homologous satellites. Then, the nucleolus in early prophase of meiosis is decidedly bigger than at somatic prophase. Since the length of the filament is a direct result of the growth and size of the nucleolus, the filament in meiosis is generally longer than in somatic mitosis. Hence the chromosomes are capable of contraction after the dissolution of the nucleolus, and the filament has therefore a chance to spiralize, as also the satellite, which thus looks bigger.

(3) Resende's (1937a) classification of species of *Aloe* on the length of filaments and presence or absence of satellite requires reconsideration in view of this hypothesis.

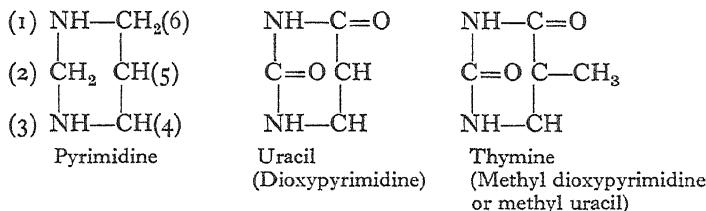
III. THE NUCLEOLUS

The nucleolus is a viscous semi-solid, denser than the karyolymph, and has a different refractive index. It is a regular inclusion of the nucleus and can be expelled by centrifuging. It is usually vacuolar and sometimes contains crystalloids. It appears early in telophase, persists through interphase, and generally disappears by the end of prophase of the next division.

Chemically the nucleolus does not seem to be universally of the same composition. Zacharias (1885) reported that the nucleolus contains proteins and plastin and no nucleic acid, while according to Unna (1928) the animal nucleoli contain globulin and nucleic acid. Zirkle (1928), by using differential fixatives, finds that the nucleolus does not contain chromatin (i.e. nucleo-proteins and nucleic acid) and he called the nucleolar material 'plastin'. He (1931) recognizes in the living cambium cells of *Pinus* two distinct substances in the nucleolus interspersed as fine droplets of different refractive indices, giving the vacuolar appearance to the nucleolus. Dermen (1933) also finds vacuolar nucleoli in the living stigmatic hair cells of *Callisia*. Similarly Heitz (1931b), using the Feulgen nucleal reaction, found no evidence for the presence of nucleic acid. Shinke and Shigenaga (1933) state from their studies on root-tips of *Vicia* and other plants that the nucleolus gives a positive reaction to lipoids, while Millon's reaction for protein is comparatively indefinite. This was confirmed by me after applying lipoid test (Lee 1937) to the nucleolus,

which does stain orange-red with Sudan III and black with 1 per cent. OsO₄. As a control, oil from Ricinus seed was used. On trying Feulgen reaction on the root-tip smear of Allium, I found no evidence for nucleic acid in the nucleolus as it was invisible without a counterstain (Mensinkai, 1939a). It appears, therefore, that the nucleolus, though mainly containing lipoids, is not devoid of nuclear proteins, which vary in quantity at different stages in the nuclear cycle, and in different organisms.

It seems necessary here to clear up some confusion about the Feulgen nucleal reaction. The reaction is popularly supposed to be a test for chromatin and to detect thymonucleic acid. Feulgen (1924) carried out the test on animal nuclei which always contain thymine in the nucleic acid to form thymonucleic acid. In plants, however, no thymine is present, but uracil instead. Both thymine and uracil have a basis in pyrimidine. Thymine is only methyl uracil in the 5 position, while uracil is a dioxyuracil with its two oxygen atoms in the 2 and 6 positions (Gortner 1929, Starling 1930, Cameron 1935). This relation can be grasped from their formulae:



Despite the absence of thymine in the nucleic acid of plant nuclei, they do give a positive Feulgen reaction. Actually this is a reaction of the monosaccharide contained in the nucleic acid. Nucleic acid is a compound of polyphosphoric acid, containing a carbohydrate (pentose) which serves to link the phosphoric acid molecules to those of purine and pyrimidine (MacLeod, 1930). In plants the carbohydrate present is *d*-ribose, in animals, *d*-ribodesose or desoxypentose; both are aldols, with the aldehyde group —CHO and are released from the nucleic acid on hydrolysis with HCl. These aldols (Levene, 1931) combine with the active principle of fuchsinsulphurous acid (Schiff's reagent), an N-sulphinic acid, with the formula R.H<^H—SO₂H (Wieland and Scheuning, 1921) to form a blue-red, almost purple, substance. The Feulgen reaction is, therefore, from the chemical point of view, a test for the aldehyde group, and since aldehyde is present only in the monosaccharide, in the nucleic acid, it is a test for the aldol carbohydrate and for nucleic acid, as aldol is a regular constituent of the nucleic acid. It is vague to say Feulgen reaction is a test for chromatin. Chromatin is a chemical substance of the chromonema and is in itself a combination of proteins and nucleic acid united as nucleo-proteins (Cameron, 1935). However, when the Feulgen reaction is

used as a test for chromatin, it may be only taken to mean a test for chromosome substance as against the nucleolar substance.

Now the two substances of the nucleolus as seen by Zirkle (1928, 1931) might be lipoids and nuclear proteins or secondary products of their interaction. This might explain its vacuolar appearance and the presence of crystalloids in it. In spite of the belief that the vacuoles are a fixation artefact, their presence in the living stigmatic hair cells of *Callisia*, as reported by Dermen (1933), seems to outweigh all contrary evidence gathered from observations on fixed material. Possibly vacuoles are exaggerated by the fixatives. No correlative increase or decrease in the volume of the vacuoles has been established; still, it is a well-recognized fact that vacuoles vary in size during the mitotic cycle. It is suggested, therefore, that the vacuoles represent droplets of a colourless liquid. These droplets seem to stand in some relation with the proteins, for in some nucleoli definite protein crystalloids are well known to occur. They may simply represent hardened final products of unhydrolysed nuclear proteins somewhat like the aleurone grains in the outer layer of the caryopsis. The crystalloids probably appear after the accumulation of the nucleolar droplets to form the finished nucleolus. This conclusion seems supported by Dermen's observations (1933) of vacuoles in the living stigmatic hair cells of *Callisia*. Observing the size relationship of the vacuoles periodically he sees no decrease in their volume and concludes that the vacuolated region is converted into true nucleolar substance by some physiological change. He further observes in one case a small 'proplastid-like body' inside the vacuoles, showing Brownian movement at the same rate as outside the nucleus.

From a consideration of these facts, Wilson's (1925) provisional classification of nucleoli as plasmosomes or true nucleoli and karyosomes or chromatin nucleoli is comprehensible, though, of course, no sharp line of distinction can be drawn, as pointed out by Gates (1932). For the nucleoli never seem to contain only protein nor are they entirely without it, though mainly made up of lipoids.

(a). *The organization of the nucleolus.*

Prior to 1924 it was a known fact that the nucleolus arises in telophase and disappears by the end of prophase of the following division; but the mode of its organization was first discovered by Van Camp (1924), who, after using Ehrlich Biondi differential stain, describes the nucleolus as arising in telophase by the flowing together of small nucleolus-like globules. In 1931 Marshak finds a close relationship between the chromosome matrix and the nucleolar substance and assumes its organization to be similar to that suggested by Van Camp. On the other hand, Heitz (1931a), after a study of thirty-three species of *Vicia*, finds that the nucleolus arises from a particular pair of chromosomes in the genome and at a particular locus, the filament or the secondary constriction. In twenty-six species he finds satellites, and only

secondary constrictions in the rest. Heitz, therefore, postulated that all plants must have satellites or secondary constrictions from which nucleoli arise; the nucleoli correspond to the satellites in number and position, and their size to the length of the satellites (more accurately SAT-filaments) from which they originate. Heitz assumes that the nucleolus arises all over the SAT-filament. He also found (1931b) in *Vicia faba* and *V. monanthus*, which are diploids with two SAT-chromosomes, two cases where a small extra nucleus having a nucleolus was present along with the two daughter nuclei. This led Heitz to conclude that even when the SAT-chromosomes are absent the nucleoli will still arise. It is a well-known fact that micronuclei with only a single chromosome can still produce a nucleolus.

However, McClintock (1934) discovered in *Zea Mays* that the nucleolus arises not on the SAT-filament but by the activity of a particular body situated at the end of a chromosome, which she calls the nucleolar organizer. McClintock assumes that the satellite stalk arises secondarily as the nucleolus grows. In X-rayed material she found a case where this organizer was broken into two portions and reciprocal translocation had taken place at this locus. Plants heterozygous for the interchange developed three nucleoli and plants homozygous for the interchange developed four nucleoli in their somatic telophase.

Another significant observation was that the functional capacity is not the same for both the segments of the severed organizing body, as the nucleoli developed from the segments were of unequal size. Also when the chromosome with the nucleolar organizing element of slower functional capacity is present without the other chromosome of higher functional capacity, the nucleolus organized was just as large. This point, besides proving the reality of an organizer, showed that there was competition between the organizers in collecting the nucleolar material.

Further, when a chromosome complement was deficient for the region of chromosome VI of maize, which includes the nucleolar organizer, as produced from the X-rayed P.M.C. and studied in acetocarmine preparations, the nucleolus is formed in telophase as an accumulation of nucleolus-like droplets along the length of each chromosome. A more striking observation was that genomic deficiency, even when the organizer is present, may lead to failure of the nucleolar material to collect into an organized nucleolus. This conclusion was arrived at from a study of the type-4 spore heterozygous for the interchange, which produced one normal nucleolus in contact with the SAT-chromosome and also nucleolus-like droplets on chromosomes, as inferred from prophase configurations. She further observes a correlation between the release of the matrix from the chromatin and the formation of the nucleolus in anaphase-telophase I, II and also in the first division of the pollen grain mitosis. McClintock therefore concludes that the nucleolus is organized from the matrix and that complete release of the matrix from the chromatin is necessary before the latter can function properly in metabolism.

Fernandes (1936) slightly modifies McClintock's hypothesis to explain the tandem condition of the satellites in *Narcissus*. He suggests that the nucleolar-organizing body (nucleologenic region) becomes active only at the ends, so that its central portion appears like the second satellite in the tandem condition. This view does not seem applicable to the satellite condition described above in *A. Cepa*. On this hypothesis the granules on the SAT-filament, which will have to be regarded as nucleolar-organizers, would show constancy of size. Actually, they vary (Figs. 23, 27-31, 34). Matsuura (1938) advances a very different hypothesis on the nucleolus organization. He observes in *Trillium kamtschaticum* nucleoli arising at both ends of chromosome 'A' and at the distal end of chromosome 'E' on the short arm. He finds neither satellites nor secondary constrictions. His photomicrographs, however (Figs. 5 and 6, p. 58), show the supposed nucleolar remnants at only one end of chromosome 'A'—on the long arm. From the extremely small size of the satellites observed by me in *T. sessile* (Mensinkai, 1939a) it is very probable that they might have missed detection in *T. kamtschaticum*.

Gates (1938) makes a suggestion similar to that of McClintock, that normally the nucleolus arises from the matrix substance and gets accumulated at an organizer, probably after undergoing some transformation to form the growing nucleolus.

Among the species examined by me for nucleolar organization only four yield information on this point—*A. Cepa*, *A. giganteum*, *A. sativum*, *A. Wallichianum*.

A. Cepa var. Egyptian shows two pairs of nucleoli in telophase. By employing Altmann's aniline-fuchsin-picric acid method it was possible to determine the nucleolus arising at the satellite region, in telophase (Fig. 35). By the above technique the nucleolus is stained yellow and the chromosomes red. The four nucleoli fuse to two masses almost always, presumably because the chromosome pairs concerned in the nucleolar organization, at least the SAT-chromosomes, happen to lie very near each other (Fig. 32). The organization of the nucleolus in telophase was not observed in the pair of nucleolar chromosomes with secondary constrictions, but their relation to the organization of the nucleolus is suggestive. In one nucleus, the nucleolus was seen held firmly at the secondary constriction in a metaphase chromosome. In this variety of *A. Cepa* the nucleolus persists up to metaphase in somatic mitosis and it was obviously remaining in contact with the locus of its origin (Fig. 19).

A similar situation was observed in *A. giganteum*, a hexaploid with $2n = 48$ (Mensinkai, 1939c) and six nucleoli in telophase (Fig. 71). It has no satellites, but six secondary, subterminal constrictions (Fig. 56). Fig. 57 shows the nucleolus organized in telophase in the secondary constriction. In two nuclei in prophase the nucleolus was seen within the secondary sub-terminal constriction, obviously suggesting the locus of its organization (Figs. 58, 59).

Employing Feulgen smears of root-tips and counterstaining with orange G, it was possible to detect the nucleolus arising in telophase in *A. sativum* (Fig. 37). Like *A. Cepa*, *A. sativum* is a secondarily balanced diploid with four nucleoli in telophase (Fig. 65). In gentian-violet preparations as well the organization could be observed (Figs. 38-41). One pair of nucleolar chromosomes have submedian primary constrictions and subterminal secondary constrictions (Fig. 36 '1-1'). The other pair have both the arms of the chromosome equal, with centric primary constriction and submedian secondary constrictions (Fig. 36 '2-2'). Essentially similar observations were made in *A. Wallichianum*, a normal diploid with $2n = 16$. It has no satellites, but a pair of nucleolar chromosomes with secondary subterminal constrictions (Fig. 42). Figs. 43 and 44 show the telophase nucleus with the nucleolus at the secondary constriction and Fig. 45 illustrates the same in prophase.

In two species, *A. Bidwelliae* and *A. senescens*, however, although the actual organization of the nucleoli in telophase could not be observed, a close relation between the nucleolar number and the number of the secondary constrictions was found to exist. *A. Bidwelliae* is an allotetraploid with $2n = 28$ and possesses five pairs of chromosomes with secondary constrictions and five pairs of nucleoli (Figs. 52 and 69). Similarly *A. senescens* is an allohexaploid with $2n = 48$ and three pairs of chromosomes with subterminal secondary constrictions, in exact correspondence with the three pairs of nucleoli in telophase (Figs. 60 and 70).

One root-tip of *A. Cepa*, however, showed many nucleolus-like bodies in a preparation from material fixed in Navashin. From seven to twelve such bodies could be seen in the resting nucleus, in place of the usual two (Figs. 63 and 64). In the light of McClintock's observations, this could only be interpreted as due to certain genomic deficiencies influencing the activity of the organizer. Or the organizer might have been lost through a mutation in the primordium of this particular root.

Some indication of the presence of the organizer and its relation to the nucleolus was also suggested from the study of the meiotic chromosomes of this species. Fig. 72 represents a body very similar to the nucleolar organizer depicted by McClintock in *Zea*. A small segment connecting the organizer and the pachytene thread looks thinner than the rest of the thread and is presumably under tension due to the attachment of the organizer to the nucleolus. This association between the nucleolus and the satellite region is more fully dealt with later.

It may therefore be concluded that the nucleolus arises at a particular locus situated at the end of a particular pair of chromosomes, the chromonemata of the end segment uncoiling as the nucleolus grows. The particular locus may be a specialized chromomere functioning through the activity of a gene situated either adjacent to the filament at the end segment of the chromosome or in the secondary constriction, which may be submedian or sub-terminal.

Of these three cases the satellite condition seems to be of later origin. This finds support in phylogeny. Chen (1936) reports that in some Protozoa (Opalinids) the nucleolus arises subterminally, while in others it occupies a considerable segment of the chromosome situated subterminally, and actually surrounds it. If the end portion of the chromosome with submedian nucleolar locus fragments, it results in a subterminal constriction. By further fragmentation it would form a suitably small end segment which, when the nucleolus is organized, is capable of being easily stretched, and thus transforms itself into a satellite with a filament.

Resende (1937 *a, b*) postulates that all plants must have satellites at which the nucleoli arise. This conclusion is based on a study of 151 species of Aloe, where he always finds the satellites in conformity with the number of nucleoli. On this hypothesis, it would be necessary to assume that a secondary constriction giving rise to a nucleolus had arisen through inversion of the end segment of a satellited chromosome.

This is inapplicable to Allium, as five species, comprising two diploids, *A. sativum* and *A. Wallichianum*, one tetraploid *A. Bidwelliae*, and two hexaploids, *A. senescens* and *A. giganteum*, show no satellites at all (Table I). To take one instance, *A. Bidwelliae*, which has ten such constrictions and ten nucleoli correspondingly, should, on this hypothesis, possess ten satellited segments inverted. In some of these secondary constrictions the satellite might be expected to be normally visible, if it did exist.

TABLE I

Species.	<i>n.</i>	<i>2n.</i>	Nucleolar chromosomes with satellites.	Nucleolar chromosomes with secondary constrictions.	Maximum no. of nucleoli in the species.
<i>A. sativum</i> L.	.	.	— 16	—	4
<i>A. Cepa</i> L.	.	8	16	2	4
<i>A. margaritaceum</i> Sibth. & Sm.	.	.	— 16	2	—
<i>A. darwasicum</i> Reg.	.	.	— 16	2	—
<i>A. Wallichianum</i> Steud.	.	.	— 16	—	2
<i>A. sicum</i> Ucria.	.	.	— 16	2	—
<i>A. decipiens</i> Fisch.	.	.	— 16	2	—
<i>A. ciliicicum</i> Boiss.	.	8	—	?	—
<i>A. Sewertzowii</i> Reg.	.	8	—	2	—
<i>A. nigrum</i> L.	.	8	—	2	—
<i>A. Scorzoneraefolium</i> (DC in Red.)	.	.	7 —	2	2
<i>A. amplectens</i> Torr.	.	7	—	6 (?)	—
<i>A. Bidwelliae</i> S. Watson	.	—	28	—	6
<i>A. cyaneum</i> Reg.	.	.	— 32	?	—
<i>A. Déséglisei</i> Bor.	.	.	— 32	?	4
<i>A. margaritaceum</i> Sibth. & Sm.	.	.	— 32	2	4
<i>A. senescens</i> L.	.	.	— 48	—	6
<i>A. giganteum</i> Reg.	.	.	— 48	—	6

(b) *The relation between the nucleoli and genomes.*

The nucleolus, arising as it does at a definite locus on a particular pair of chromosomes, also bears a certain definite relation to the chromosome complement it represents. A valuable contribution, in this respect, was first made by de Mol (1928). He established by studies on *Hyacinthus* diploids, triploids, and tetraploids that only one nucleolus stands for a genom or basic set of chromosomes in a species; in other words, the nucleolar number may be taken as a relative guide to polyploidy. This was found applicable to most of the *Allium* species, including eleven diploids, four tetraploids, and two hexaploids (Figs. 61–71 and Table I). The nucleoli have been drawn either from the resting stage or from telophase wherever available. Only one pair of diploid nuclei is drawn as typical of diploids, while all tetraploids and hexaploids have been represented by a drawing for each species. A deviation was observed in two diploids, *A. Cepa* and *A. sativum*, each showing four nucleoli instead of two. This is because they are secondarily balanced diploids with a duplication of a pair of nucleolar chromosomes (Mensinkai, 1939c). A similar discrepancy was met with in *A. Bidwelliae*, which is an allotetraploid with five pairs of chromosomes with secondary constrictions and five pairs of nucleoli (Figs. 52 and 69).

(c) *The behaviour of the nucleolus in prophase.*

As is obvious from the foregoing account, the nucleolus arises through the activity of an organizer at a definite locus on the chromosome. In somatic resting stage there appears no marked increase in its volume. However, in premeiotic interphase it does grow as the nucleus and cell grow, and it will be in constant association with the locus of its organization in the last telophase. The recognition of this association opened a new field of inquiry regarding the possible return of its material to the chromosomes from which it arose. Several investigators have reported this relationship both in mitosis and meiosis, beginning from 1895, when Farmer observed the nucleolus attached to the reticulum in a 'clear and unmistakable manner'. This has been dealt with in detail by Gates (1937).

The nucleolus has a different refractive index and is capable of being displaced when centrifuged and is hence denser than the karyolymph. It has been estimated that the chromosomes shorten by about ten times from interphase to metaphase. Yet the nucleolus keeps its position of origin from telophase to the end of prophase, as verified by several workers. Bhatia (1938) shows in P.M.C. of tetraploid wheat four threads attached to a single fused nucleolus in leptotene and six such threads in a $6n$ wheat. Also in pachytene the homologous threads, as they pair, may show their nucleoli fusing. These movements must overcome considerable resistance of the nucleolus as its position is shifted in the karyolymph. Its position near or attached to the satellite in prophase can therefore only mean a rigid organic contact between the satellite and itself. Again, failure of pairing of the homologous leptotene

threads at the points of attachment to the homologous nucleoli is an additional proof of such contact. This was observed in three species (Figs. 75, 120, 126, 128-9. Also occasional detachment of the satellite from the filament while the satellite remains attached to the nucleolus during meiotic prophase seems a sound proof of the firmness of attachment. (Figs. 93, 99, 107, 111, 129, 138. See also Fig. 131, where one chromosome is drawn away from its homologue and is in firm attachment with the nucleolus.) Incidentally, this failure to complete pairing must mean that the nucleoli, while capable of fusing, are stiff and not very liquid. My studies on several species of *Allium* make this point of satellitic association to the nucleolus so evident that Dermen's (1933) and Fikry's (1930) suggestions of chance association need hardly be taken as valid. It might be argued that if the association were firm, some sort of attachment-mark should be observed on the nucleolus. This seems unnecessary as the nucleolus is semi-solid when organized, and a liquid during its organization.

Seventeen species of *Allium*, including eleven diploids, four tetraploids, and two hexaploids, were studied, and all except one or two, which could not be well investigated for want of material, yield information in support of the reality of the satellitic association to the nucleolus. The attachments may be seen throughout the meiotic prophase, diploids two, tetraploids four, and hexaploids six, respectively (Figs. 72-138, comprising nine species, *A. Cepa*, *A. Sewerzowii*, *A. siculum*, *A. cilicicum*, *A. Scorzoneraefolium*, *A. nigrum*, *A. amplexens*, *A. Bidwilliae*, and *A. senescens*).

The mitotic attachments of seven species have been described in Figs. 3, 4, 19-27, 45, 47-8, 51, 53-5, 58-9. The species are *A. margaritaceum* (tetraploid), *A. Cepa*, *A. Wallichianum*, *A. decipiens*, *A. siculum* (diploids), *A. Bidwilliae*, *A. Déséglisei* (tetraploids), and *A. giganteum* (hexaploid). The rest of the species could not be studied in respect of satellite attachment to the nucleolus, for want of material.

A. Cepa seems to offer an explanation for the nucleolar budding. It has been suggested by several workers (see Bhatia, 1938) that the nucleolus buds actively, and various functions have been imputed to such 'buds'. From the chemical constitution of the nucleolus, which is mainly lipoids, it seems wholly inadequate to attribute activity to the nucleolus as to a living proteinaceous entity. At least in *A. Cepa* it could easily be gathered from the successive stages in pachytene from Figs. 77-82 that the 'bud' is merely a stage in the progressive fusion of the homologous nucleoli. In Fig. 77 the nucleoli are almost equal, while with the other figures one goes on diminishing and the other increasing in size. Pachytene being the stage of longest duration in meiosis, complete fusion is generally over by the end of it. Appearance of budding may also be due to differential rate of activity of the homologous organizers. This seems unlikely, however, as the nucleoli of a pair are usually of the same size in premeiotic resting nuclei. On this hypothesis, therefore, the 'bud' in Figs. 95 and 118 can only be explained as due to

movement of the chromosomes from their points of attachment, during pairing.

A. amplexens, though a diploid with $n = 7$, departs from the normal nucleolar number. Fig. 125 is a resting P.M.C. with six nucleoli. Figs. 127 and 129 show them fused in pairs at the satellite ends of the bivalent chromosomes. Fig. 130 (diplotene) shows one bivalent well in contact with the nucleolus, the other being very near it, though not attached. The nucleolus is very small, the other two also seem to have disappeared already, and it cannot be said with any certainty if both the bivalents were attached to the nucleolus, one becoming detached afterwards. Fig. 133 shows pollen grains with three and four nucleoli, respectively. As no further study of this species could be made for want of material, no attempt has been made to explain this condition.

(d) *The function of the nucleolus.*

So numerous have been the functions assigned to the nucleolus (Ludford, 1922; Wilson, 1925; Sharp, 1934) that only a few of the important ones will be taken for consideration, mostly from Zirkle (1928). (1) It has been held that the nucleolus is an accumulation of waste products of the nucleus and is periodically fragmented and passed out into the cytoplasm. (2) The nucleolus carries the excretory and secretory products out from the nucleus to the cytoplasm. (3) The nucleolus is a storehouse of chromatin of the resting nucleus. (4) The nucleolus is a storehouse of nucleic acid, to be drawn upon as the chromosomes resume their basophilic character. (5) Fragments of the nucleolus pass into the cytoplasm of the animal egg and give rise to yolk granules. (6) Nucleolar globules are bearers of stimulating or finishing material of the genes. (7) The nucleolus is a reserve product of metabolic activity and is used in the upbuilding of certain parts of the cell. (8) In certain insects the nucleolus contributes to the activity of the silk glands. (9) The nucleolus forms the blepharoplast (Yuasa, 1938).

The very fact that the functions of the nucleolus have been so varied goes to show the plasticity of function, many of which the nucleolus might well be adapted to, secondarily, in different cells, in different organisms, and under different circumstances. Its universal presence, however, in all the living cells of all organisms, plants, and animals, except the very lowest, must mean some common function in the economy of life.

It is becoming increasingly evident that the chromosome is an aggregate of sub-units called genes, although there is diversity of opinion as to whether genes are 'delimited entities' or 'ultramicroscopic conceptions' of localized positions on the thread. The chromosome itself behaves as a unit as it reproduces itself and has its life-cycle in relation to the other chromosomes. The chromosomes, therefore, behave collectively as individuals in the cell, with the nucleus and cytoplasm.

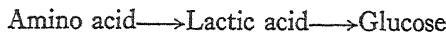
Since the life processes are essentially the same in all living organisms, the

basic principles governing life will therefore apply to the life-cycle of the chromosomes. And if the chromosomes are regarded in this light, the function of the nucleolus seems easy to comprehend.

As is indicated by the foregoing account, the nucleolus appears to arise from the surface of all the chromosomes of a genome in the form of fine droplets which pass through the karyolymph and collect at the organizer. In telophase, interphase, and prophase it remains in contact with the locus of its organization. Physically it is denser than the karyolymph and has usually a few vacuoles and sometimes crystalloids. Chemically, it is proved to contain mainly lipoids, and nuclear proteins at times.

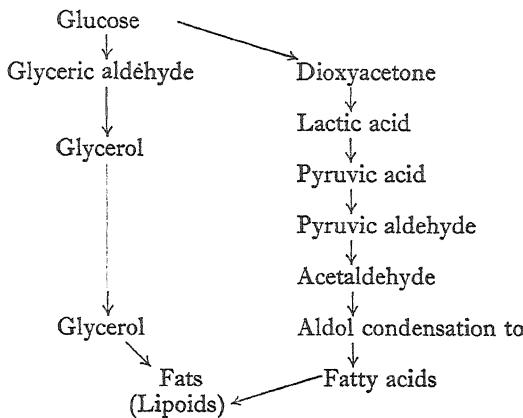
On the other side, the chromosome from which the nucleolus arises is morphologically distinguishable, at least at some stage, into a chromonema as its main constituent with an envelope of matrix. The chromonema contains mostly nucleoproteins united with nucleic acid, along with some lipoids (Shinke and Shigenaga, 1933). The matrix has lipoids as its main constituent free or partly combined to form lipoproteins. In prophase the chromosomes are chromatic with very little free matrix, the chromaticity increasing with the advance of prophase until telophase, when the matrix becomes apparent. As Alexander and Bridges (1928) suggest, the matrix seems to arise from the nucleoproteins by the chromatin-hydrolysing enzymes. Biochemistry throws considerable light on this point. Although the metabolism of the nucleoproteins, in animals, is not thoroughly understood, the formation of lipoids from the nucleoproteins is known. According to Cameron (1935), the nucleoproteins exist as 'salts of nucleic acid united in varying amounts, in virtue of their acid properties, to one or more molecules of protein in virtue of their basic properties'. The union is of such a type that it can be easily broken by enzymes. When hydrolysed they yield nuclein, a monoprotein-nucleotide whose protein radical is a protamine or histone, and nucleic acid. Further hydrolysis breaks up nuclein into its protein and nucleic acid. The nucleic acid is a complex acid commonly known as yeast nucleic acid in plants and thymo-nucleic acid in animals. It is composed of phosphoric acid linked with the bases of purine and pyrimidine, by a carbohydrate. On hydrolysis it yields (1) phosphoric acid, (2) a pentose, (3) and (4) two purine bases guanine and adenine, amino derivatives of purine, and (5) and (6) two pyrimidine bases, cytosine and uracil.

The protein of nucleoproteins is an aggregate of amino-acids joined end to end. On hydrolysis, the amino-acids, all those having a straight chain of C atoms, yield glucose probably as follows:



(Gortner, 1929, and Cameron, 1935). It is from glucose so formed that lipoids have been found to arise by another series of complex reactions. The probable course of transformation is briefly that glucose gives rise to glycerol

and fatty acids, which combine to form fats and lipoids. The process is schematically represented below.



Thus it will be seen that the lipoids arise from the protein constituent of the chromatin or the nucleoproteins, and not from nucleic acid, although it contains pentose.

How far this set of reactions of lipid formation from chromatin applies to plants cannot be stated with certainty. However, there is indirect evidence in support. The presence of chromatin hydrolysing enzymes (nucleases) in plant nuclei is recognized (see reviews of Mathews; Alexander, and Bridges, 1928). This makes the hydrolysis of nucleoproteins to nucleic acid and to protein comprehensible. Also evidence is not wanting in showing the existence of nucleic-acid-hydrolysing enzymes. As in animals, the end products of nucleic-acid hydrolysis are the same as given above with the only difference that *d*-ribodesose is replaced by *d*-ribose and thymine by uracil. Only the point whether lipoids arise from the protein of the chromatin as in animals is not experimentally confirmed. That the matrix arises from the chromatin is clear from the foregoing account and that it consists of lipoids is ascertained from the microchemical tests (Shinke and Shigenaga, 1933) of the chromosomes. It is also easy to see, by a process of elimination, that these lipoids must come from the protein of the chromatin and not nucleic acid. The end products of nucleic acid have been shown to contain no fats, either in plants or animals (Cameron, 1935). Since in animals the hydrolysis of chromatin and its other constituent, nucleic acid, is enzymatic and since lipid formation from proteins is similarly an enzyme action, it may be taken that lipoids are formed in plants likewise, from proteins of the chromatin and presumably by a similar series of reactions. The matrix, therefore, arises directly from the nucleoproteins themselves and does not seem to be a product of interaction between the chromosome and karyolymph developed at the chromosome surface, as held by Marshak (1931). And seen as it is on the surface of all the chromosomes in a genome, it is likely to have material from all the genes.

Likewise, the nucleolus is mostly lipoids with some nuclear proteins at times. Like the matrix, it arises from all over the chromosomes, though organized into a growing mass at a definite locus on the chromosome thread, and has material from all the genes. The proteins it contains might simply represent unhydrolysed proteins of the chromosomes. Thus there appears a very close relationship between the matrix and the nucleolus in their composition, distribution, and origin.

Also, the time of appearance of the nucleolus in the mitotic cycle has an important bearing on the metabolic activity of the chromosomes as measured in terms of spiralization and despiralization. In respect of this one function of the proteins, namely, despiralization, it can be seen (Mensinkai, 1939a) that the protein activity is minimum at metaphase, their pH then being very near the I.E.P. and the charge nil. As there is no appreciable change in the size of the chromosomes from metaphase to the end of anaphase the nucleo-proteins may be taken to be neutral at this time, their positive charge balancing the negative charge. During metaphase-anaphase the chromosomes are therefore metabolically inactive. With the commencement of telophase, they get hydrated and charged and become active, with a change in their pH relationships, and the chromonemata elongate. This activity is perfectly correlated with the organization of the nucleolus at this stage, which exudes from the body of all chromosomes and becomes visible at the organizer.

In consideration of these two relationships between the nucleolus and the metabolic activity of the chromosomes on the one hand and the matrix on the other, it seems quite certain that the nucleolus is a product of exudation formed during the normal metabolism of the chromosomes, as was barely suggested by McClintock (1934). This finds support in Kuwada's (1927) experiments of artificial unravelling of the chromonemata in the P.M.C. of *Tradescantia* by exposure to ammonia. Although the superficial appearance of the resting nucleus was brought about, no nucleolus could be seen. Further, the frequent formation of the nucleolus and its extrusion in active cells, such as gland cells and nerve cells, as also the absence of the nucleolus in inactive cells (Ludford, 1922), such as the undifferentiated cells of the germinal epithelium, point to the same conclusion.

The cause of nucleolar accumulation, however, is still in the region of speculation. The best available suggestion is the unspecific metabolic activity of the organizer, as defined by McClintock (1934). The evidence for such activity is that when the organizer is absent or influenced by genomic changes it ceases to function and the nucleolar substance is scattered in droplets instead of being organized into a finished nucleolus.

A solution may be sought in a chemical consideration of the problem. I suggest that the nucleolar particles containing, as they do, mainly lipoids, are electrically attracted to the organizer, which might behave as a pole with an opposite charge. It is true that pure lipoids are non-electrolytes, but if a protein layer be supposed to envelope the globules, they can get charged.

Whether they have such a layer or not, need not invalidate the fact that fat globules, as in milk, can bear a charge *opposite* to that of the milk (protein). If it is basic, i.e. above pH 4.5, which is the I.E.P. for milk, the fat droplets are negatively charged and migrate to the anode; if it is acid, i.e. pH below 4.5, they go to the cathode, being positively charged (Seifriz, 1936).

The nucleolus never containing lipoids exclusively suggests the possibility of such a stabilization membrane of protein. It is also becoming more evident that the whole course of mitosis is electrically determined by the pH changes and the electromotive force developed thereby. The chromosomes in themselves are negatively charged. The nucleolar particles as they leave the surface of the chromosomes in the form of matrix will therefore be assumed to be positively charged. It also follows that the organizer has an intensity of charge greater than at any point on the chromosome. How far this is true is a matter for more research. Some support may, however, be taken from the fact that the centromere is most strongly charged during the anaphase separation of the chromosomes. To explain the accumulation of the nucleolus, however, would require the assumption that the local negative charge on the nucleolar body is very intense in comparison with that on the surface of the chromosomes.

Simultaneous to the exudation of the nucleolar material from the chromosomes in telophase, the one visible change is in their chromaticity, which is practically uniform under all fixatives. Hence achromaticity, loss of matrix and its aggregation into a localized mass, the nucleolus, all act simultaneously. When the prophase begins the chromosomes become dehydrated and chromatic, and the nucleolus begins to wane simultaneously. Hence it is not unlikely that the matrix material contained in the nucleolus is redistributed to the emerging chromosomes. A similar suggestion was made by McClintock (1934). The organizer, in view of its firm contact with the nucleolus until the disappearance of the latter, probably absorbs some of the nucleolar material and passes it on to the chromosome of which it is a part, while the rest of the nucleolar material may go back to the other chromosomes. Biochemically, the absorption of the nucleolar material in this way is the incorporation of lipoids into the chromosomes of the succeeding prophase. Among animals the catabolism of fat consists in the formation of CO_2 and H_2O or in the formation of glucose from one of its products of hydrolysis, or presumably both. Amino-acid formation from glucose and the building up of proteins therefrom is also possible chemically and an ascertained fact in animals (Cameron, 1935). Although this process cannot be warranted to take place in plants it very probably does. When fat is treated with esterase (fat hydrolysing enzyme) it will always split into glycerol and fatty acids, whether the enzyme used be from animals or plants. The glycerol so formed, as also the fatty acids, are generally oxidized by a series of breakages in the C-atom chain, eventually to CO_2 and H_2O . Along with this process some glycerol is also converted to glucose (Cameron, 1935), as a chemical reaction in an organism is never one-sided. Glucose, being soluble, can be conceived to pass through

the karyolymph to the chromosomes and be incorporated within them. The fate of glucose after its absorption as such can only be inferred. It may be tentatively suggested that it is used up in the formation of nuclear proteins of the emerging chromosomes and in giving them the chromaticity so characteristic of this stage. The glucose so formed from glycerol is easily convertible into alanine, an amino-acid, *via* pyruvic aldehyde (Gortner, 1929, and Cameron, 1935). Amino-acids being the 'building stones' for the protein molecule, it is not unlikely that alanine so formed may form part of the nucleoproteins of the chromonemata of the prophase chromosomes. The fact that the chromonemata contain nucleoproteins and are chromatic, coupled with the fact that they are achromatic at the time of this absorption, seem strikingly suggestive of the utilization of the nucleolar material for the revival of chromaticity.

In view of these considerations, one of the functions that could be attributed to the nucleolus is that it may act as a fuel substance in the nucleus and it may also help in developing chromaticity of the emerging prophase chromosomes. However, what appears to me more important is that it is a mistake to regard the nucleolus as a special body destined for some definite function. If we start by considering how the chromosomes metabolize, the nucleolus and the place it holds in the economy of the cell are better revealed. The nucleolus, from this point of view, appears to be just a regular product of the normal metabolism of the chromosomes. The one outstanding feature of the chromosome metabolism is that it results in plentiful release of chemical energy. For during this process of nucleolar appearance the chromatin is hydrolysed to nuclein and nucleic acid; nuclein to histone or protamine, and nucleic acid; the protein itself breaks down to amino-acids which form glucose, from which arise lipoids by a series of reactions to appear in droplets and accumulate as nucleolus. Simultaneously the nucleic acid hydrolyses to phosphoric acid, *d*-ribodesose, and bases of purine and pyrimidine, which may undergo further disintegration. All these steps of chemical transformations of complex compounds to simpler substances must mean liberation of abundant, utilizable energy, which might very well serve as the *basic source of energy for all the activities of the cell, in particular, and for the organism in general*. The nucleolus, too, seems to participate in this process of energy-supply, though not in full.

The fate of the end products of the nucleic acid is not known. Evidence is accumulating, however, that although no uric acid appears in plants as in animals, the two metabolisms have many points in common. In coffee and cacao it is believed that these bases of purine and pyrimidine serve as precursors of caffeine and theobromine, the active principles of the plants referred to. And purines can all be regarded as xanthine derivatives which are widely distributed in the Phanerogams (Gortner, 1929, and Cameron, 1935). This, incidentally, shows the presence of nucleic-acid-hydrolysing-enzymes in plant nuclei.

With this outlook on the position and nature of the nucleolus during the

chromosome metabolism, the functions ascribed to it, strange though they may seem, do not appear inconceivable. They appear to be mere adaptations of the cells and the tissues concerned, in using the lipid material or the nucleoprotein material accumulated in the nucleolus, for special functions. The view that the nucleolus represents a mere ergastic substance seems invalidated, as the nucleolar material appears not only to be absorbed but also used as a source of energy; especially since the reality of organic connexion between the chromosome and the nucleolus is a proven fact.

The persistence of the nucleolus may be accounted for as the extra amount of lipid material left after utilization by the chromosomes and the cytoplasm up to the metaphase of the nuclear division. For the nucleolus is found up to metaphase, only in species which are not normal in their nucleolar number, having always a superfluous amount of the nucleolar material. The hypothesis that the nucleolus carries the finishing or stimulating material of the genes, in the form of enzymes for the cytoplasm, seems less probable, as the enzymes, as held by Alexander and Bridges (1928), are permeable through the nuclear membrane.

IV. SUMMARY

1. The satellite appears to be the rolled end of the chromosome chromonemata.
2. The SAT-filament is a continuation of the chromonemata and is a spiral of an order lower than the minor spiral. It is also chromatic, containing thymo-nucleic acid like the rest of the chromonemata.
3. Tandem satellites with two-four granules on the SAT-filament were observed in an Egyptian variety of *Allium Cepa* and have been interpreted as twists (gyres) of the chromonemata, fused in fixation. They do not seem to be chromomeres.
4. It is attempted to explain the larger size of satellite in meiotic prophase as compared with somatic prophase, and also the usual disappearance of the satellite in metaphase I in meiosis, although it is visible in somatic metaphase of the same species.
5. The satellite seems to be higher in the scale of evolution and is postulated to have arisen from a more primitive condition, the secondary constriction.
6. The number and generally the origin of the nucleoli in somatic telophase was observed in seventeen species of *Allium*.
7. The hypothesis of McClintock about the organization of the nucleolus is confirmed, and a body similar to the nucleolar organizer was observed in the pachytene stage of *Allium Cepa*, (Egyptian variety). The nucleoli arise either in the secondary constrictions or adjacent to the satellite, by the activity of the nucleolar organizer. The number of nucleoli for a species was found to correspond to the number of the nucleolar chromosomes, except in four species, diploids generally having two nucleoli, tetraploids four, hexaploids six.
8. It is suggested that the nucleolus organizer might behave as a charged pole in collecting the oppositely charged nucleolar particles from the

chromosome surface. The lipid particles (of the nucleolus) are supposed coated with a stabilization layer of protein which enables them to get charged.

9. The nucleoli were observed as two different pairs for allotetraploids and as three different pairs for allohexaploids. Only in one species, *A. Bidwelliae*, a tetraploid, the nucleolar number was ten, instead of four. An explanation of this case is suggested.

10. The nucleolus contains mainly lipoids, with nuclear proteins at times.

11. It is organized probably as a result of the hydrolysis of the chromatin by chromatin hydrolysing enzymes. The lipoids it contains are presumably derived from the protein part of the chromatin by enzyme action.

12. The normal metabolism of the chromosomes results in the release of abundant chemical energy which very probably serves as the basic source of utilizable energy in the cell economy in particular, and the organism in general.

13. It is conclusively shown that the nucleolus remains firmly attached to its locus of organization or near it, from the time it is organized until the time of its disappearance.

14. "Budding" of the nucleolus is explained as a mere stage in progressive fusion of the homologous nucleoli.

15. It is hypothetically suggested that the nucleolus acts partly as a fuel substance in the cell economy and partly in reviving the chromaticity of the emerging chromosomes, by its absorption and incorporation into the nucleo-proteins.

V. ACKNOWLEDGEMENTS

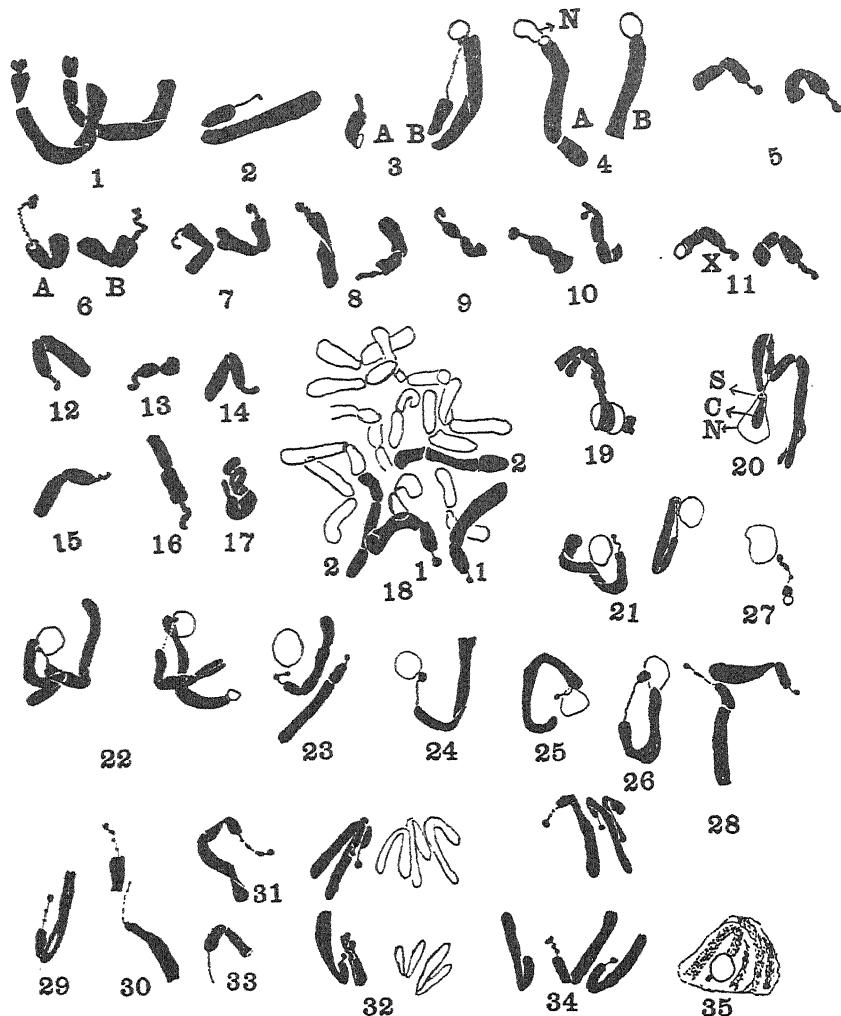
It is with gratitude I express my sincere indebtedness to Professor R. Ruggles Gates for his very helpful guidance in many ways, and for his never-failing kindness, in the course of the work.

I also thank the Navalgund Sirsangi Trust, Belgaum, Bombay, for affording me facilities to undertake this work in the University of London.

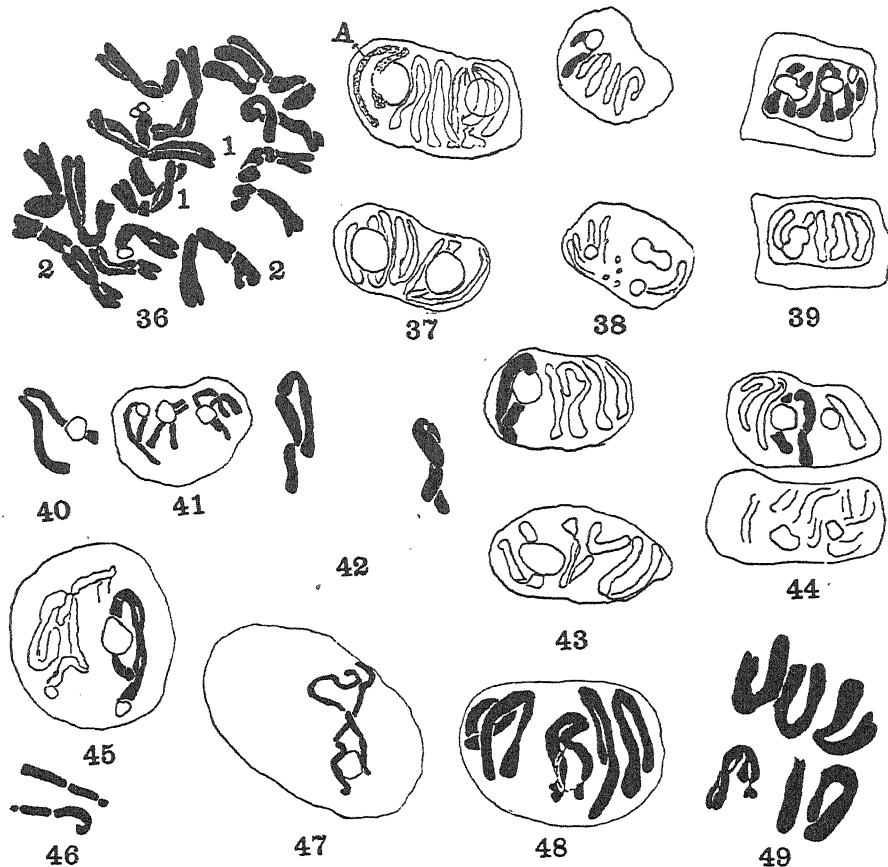
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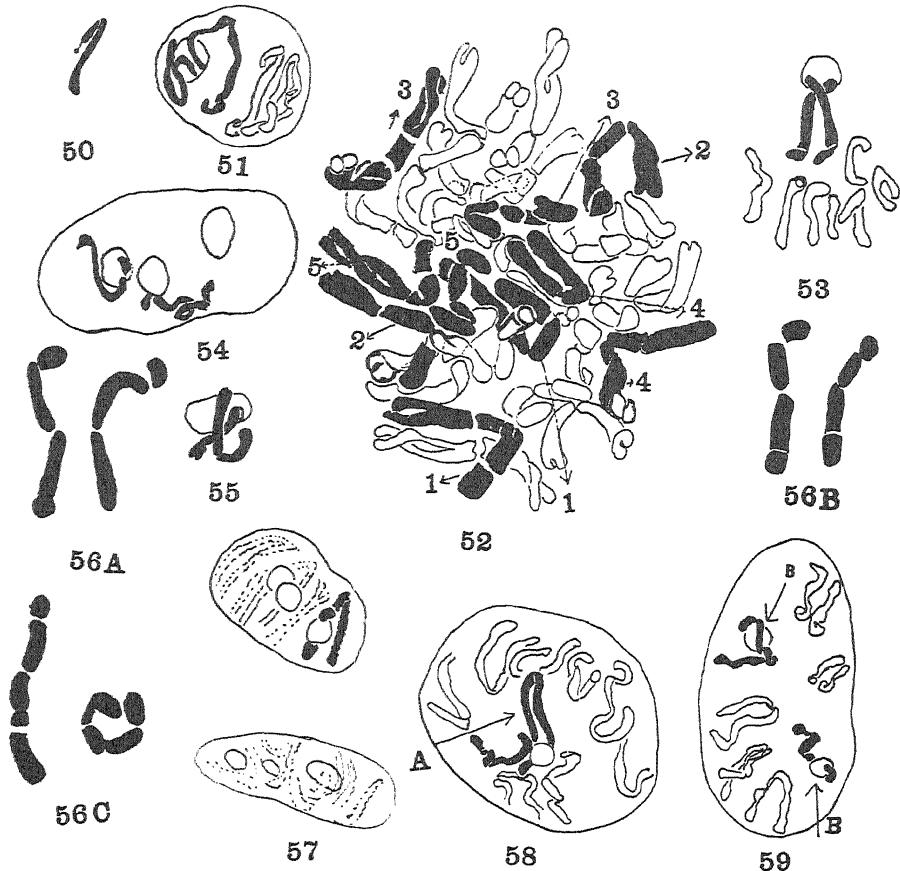


Figs. 1-35. Mitosis: *A. margaritaceum*, $2n = 32$, Figs. 1-4, and *A. margaritaceum*, $2n = 16$, Figs. 5-17, are from root-tips fixed in Benda. Figs. 1-4. Metaphase chromosomes showing the normal pair of SAT-chromosomes in Fig. 1 and the absence of satellite in others, and the attachment to the nucleolus by only a thread. In Fig. 4 the attachment is by long arm, presumably at a subterminal constriction. Fig. 5. A pair of anaphase SAT-chromosomes. Figs. 6-10. Metaphase SAT-chromosomes showing variation in thickness of thread and size of satellite. Figs. 11-17. Anaphase chromosomes without satellites. At X in Fig. 11 the tip of the filament appears like a satellite due to the curved end of the filament. *A. Cephaelis*, $2n = 16$, Figs. 18-35, fixed in 2BE after Altmann's aniline-fuchsin-method. Fig. 18. Metaphase plate, showing a pair of SAT-chromosomes (1-1) and a pair of chromosomes with secondary submedian constrictions (2-2). Fig. 19. Metaphase chromosome with the persisting nucleolus held at the secondary submedian constriction. Fig. 20-2. A pair of metaphase chromosomes attached to a common nucleolus (Fig. 20) and to two separate nucleoli (Figs. 21-2). Note the reduced size of the satellite and a longer SAT-filament. In Fig. 20 S = satel-



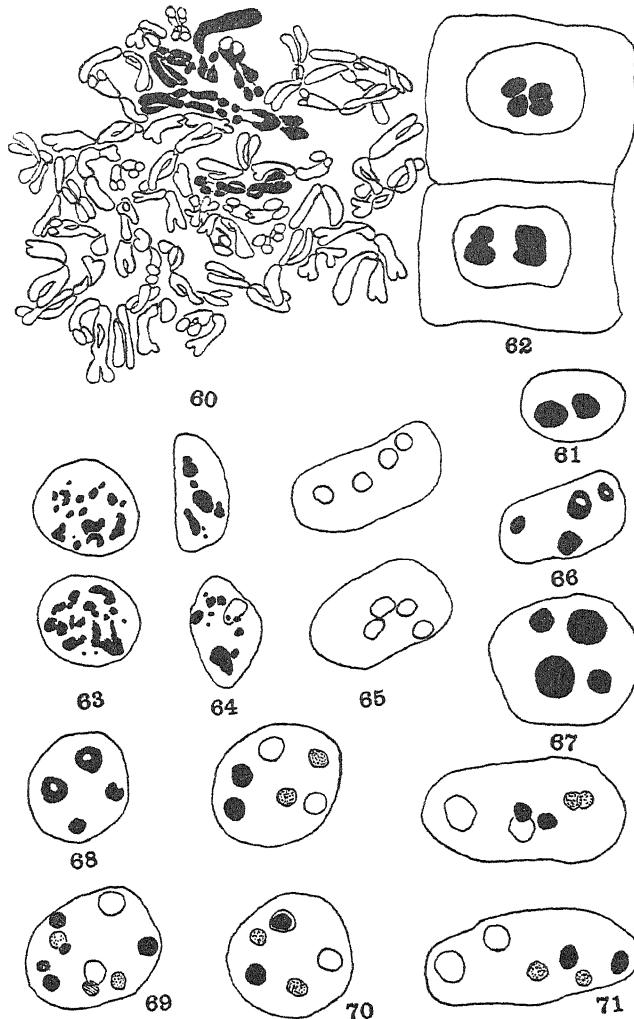
lite, C = chromosome end, N = nucleolus. Figs. 23-7. A single metaphase chromosome attached to or lying near a nucleolus and showing variation in the satellite size. In Fig. 23 the satellite appears tandem. In Fig. 27 the SAT-filament seems pulled out and the gyres of the chromonemata appear fused to give rise to a thick filament and a granule (cut chromosome). Fig. 28-31. Metaphase chromosomes with 1-4 granules on the SAT-filament. Fig. 32. Anaphase with unequal pair of satellites. Fig. 33. A cut SAT-chromosome without any satellite. Anaphase. Fig. 34. Anaphase with two pairs of S_1 and 'tandem' satellites. Fig. 35. Telophase showing the organization of the nucleolus at the satellite constriction.

FIGS. 36-49. *Mitosis*: *A. sativum*, $2n = 16$. Figs. 36-41. Fig. 36. Metaphase plate with two pairs of nucleolar chromosomes with secondary submedian constrictions (1-1 and 2-2). Root-tip smear fixed in alcohol acetic 3:1 and stained in Feulgen. Figs. 37-41, showing the organization of the nucleolus in telophase. Chromosome A in Fig. 37 corresponds to (1-1) chromosomes in Fig. 36. Root-tip smear fixed as in Fig. 36 and counterstained with orange G. Figs. 38-41 are from root-tips fixed in 2BE and stained in gentian violet. Chromosome in Fig. 40 corresponds to chromosomes (2-2) in Fig. 36. *A. Wallichianum*, $2n = 16$. Figs. 42-5. Fixed in Navashin (Karpechenko) and stained in gentian violet. *Mitosis*: Fig. 42. Metaphase side view with a pair of nucleolar chromosomes with secondary submedian constrictions. Figs. 43-4. Telophase chromosomes showing organization of the nucleolus. Fig. 45. Prophase chromosome with the nucleolus in the secondary constriction. The nucleolar chromosomes in Figs. 43-5 correspond to the pair in Fig. 42. *A. decipiens*, $2n = 16$. Figs. 46-8.

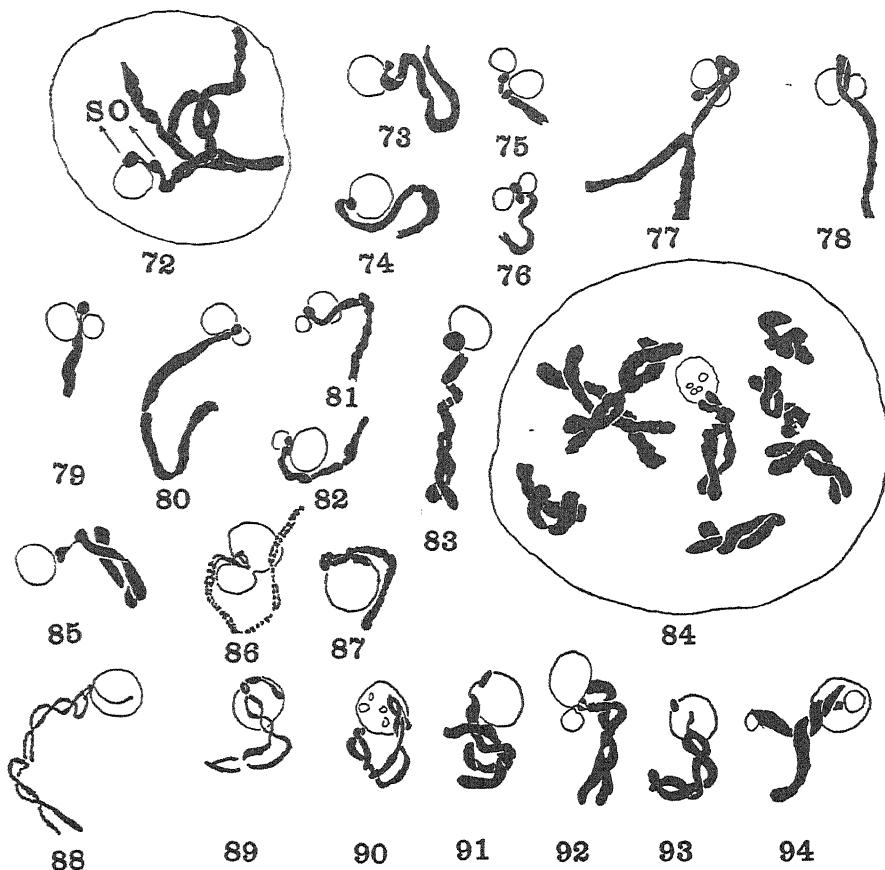


Root-tips fixed in Navashin. Fig. 46. A pair of metaphase SAT-chromosomes. Figs. 47-8. Prophase with two chromosomes (Fig. 47) and (Fig. 48) one chromosome attached to the nucleolus. *A. siculum*, $2n = 16$. Fig. 49. Metaphase with a SAT-chromosome. Root-tip fixed in Benda.

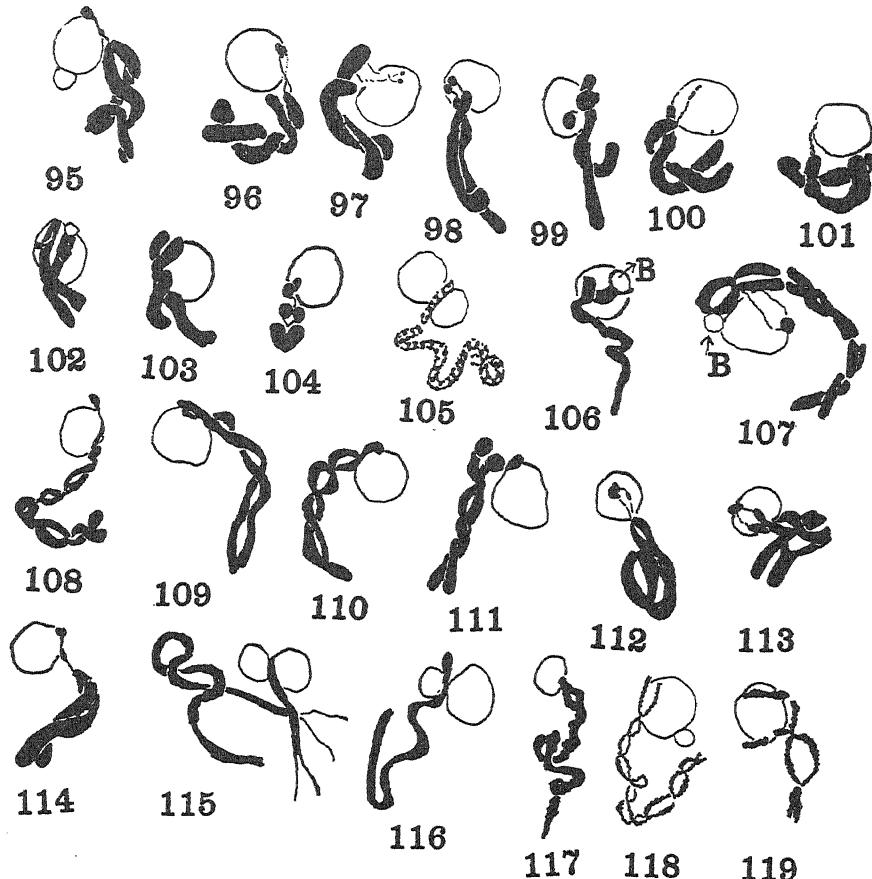
Figs. 50-9. Mitosis: *A. siculum*, Figs. 50-1. Root-tips fixed in Benda. Fig. 50. Anaphase with a SAT-chromosome. Fig. 51. Prophase with a pair of chromosomes attached to the nucleolus. *A. Bidwelliae*, $2n = 28$. Figs. 52-3. Root-tips fixed in modified 2BE and Benda respectively. Fig. 52. Metaphase plate with twenty-eight chromosomes and five pairs of nucleolar chromosomes with secondary constrictions. Fig. 53. A pair of metaphase chromosomes attached to the nucleolus. *A. Déséglisei*, $2n = 32$. Figs. 54-5. Root-tips fixed in Navashin. Fig. 54. Prophase with a pair of chromosomes attached to two separate nucleoli at the subterminal constrictions. Fig. 55. A pair of metaphase chromosomes attached to a single nucleolus. *A. giganteum*, $2n = 48$. Figs. 56-9. Root-tips fixed in Levitsky 1:1. Fig. 56. Three pairs of nucleolar chromosomes from metaphase. Fig. 57. A telophase chromosome showing the organization of the nucleolus in the secondary constriction. Figs. 58-9. Prophase chromosomes marked A in Fig. 58, B in Fig. 59, with the nucleolus in the subterminal constriction.



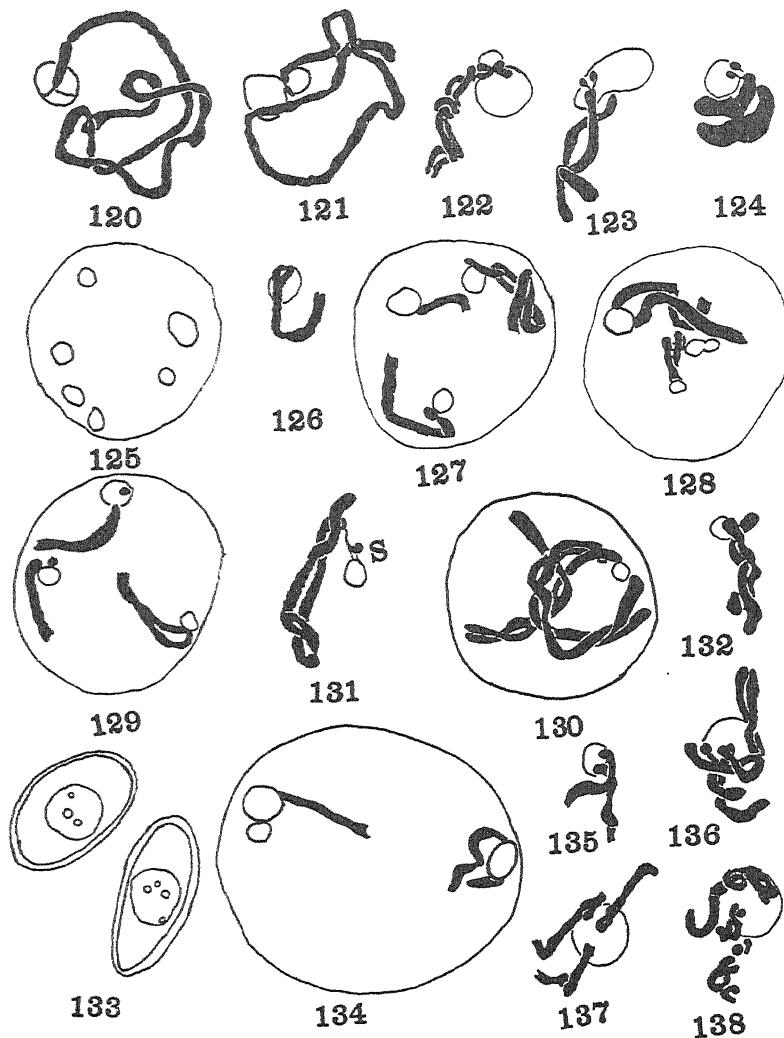
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Figs. 95-119. Figs. 95-104, P.M.C.s fixed in 2BD. Fig. 95. A bivalent with satellite attached to two nucleoli in fusion and with a granule on the filament. Figs. 96-7. Same stage as in Fig. 91. Note the small size of the satellites and the long, fine SAT-filaments. Fig. 98. Diakinesis. A bivalent attached to the nucleolus with a pair of satellites. Fig. 99. Same as in Fig. 93. The satellite detached. Figs. 100-1. Diakinesis. Mark the presence of a thread and the absence of a satellite. Fig. 102. Diakinesis with only one satellite visible. Fig. 103. Late diakinesis. A bivalent attached to the nucleolus without a satellite. Fig. 104. Late diakinesis with the normal pair of satellites attached to the nucleolus. *A. siculum*, $n = 8$, Figs. 105-14 showing attachment to the nucleolus. P.M.C.s fixed in Benda. Fig. 105. Pachytene. A pair of threads attached to two nucleoli. Fig. 106. Pachytene. Failure of fusion of threads. Note the 'bud' B. Figs. 107-8. Diplotene. Note the detached satellite with the filament and the bud B in Fig. 107. Note also the small size of satellite in Fig. 108. Figs. 109-14. Diakinesis. Note detached satellite in Fig. 111, and thickening on one filament in Fig. 112. Fig. 114. Late diakinesis. Note thickening of satellite thread. *A. cilicicum*, $n = 8$, Figs. 115-19. P.M.C.s fixed in 2BD. Fig. 115. Zygote. Fig. 116. Pachytene. Figs. 117-19. Early, mid, and late diplotene. Note the attachment at secondary constriction in Fig. 119.



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Macroconidial Formation in *Ophiostoma majus* (van Beyma) Goidanich

BY

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With Plate XXIV

INTRODUCTION

DETAILED studies of endoconidial formation have been made in the genera *Thielavia* and *Sphaeronema*. Work has been concentrated on microconidial formation, and the formation of the closely related macroconidia has not received equal attention. The investigation described in this paper was undertaken when it was found that macroconidial formation in the *Thielavia* stage of *Ophiostoma majus* differed considerably from the methods previously described for this and other fungi.

Zopf (1876) gave the first description of endoconidial formation in *Thielavia*. He describes both exogenous chlamydospores and microconidia in *T. basicola*. The latter are formed in 'acropetal' succession at the end of the conidiophore, and their wall on dehiscence splits into two layers. The outer wall forms a sheath, the inner the wall of the conidium. Von Höhnel (1904) recognizes two sizes of spore in *T. paradoxa*, but as all stages of transition between the two are seen, he considers the macroconidia to be mature microconidia. They are formed by septation of the inner wall of the conidiophore, after the dissolution of the tip of the outer wall. Gilbert (1909) describes the formation of microconidia in *T. basicola* as taking place within the conidiophore: liberation is by the bursting or dissolution of the apex of the wall, continued growth of the protoplast producing a succession of spores. Duggar (1909) states that the microconidia are formed in 'basipetal' succession within the conidiophore, and that macroconidial formation differs only in size. According to Brierley (1915), the microconidia are abjoined in 'acrogenous' succession from the end of the conidiophore. The wall of the young spore splits into two layers, the outer forming a sheath, the inner the wall of the conidium. The sheath is ruptured at the apex on dehiscence. Cases of macroconidial formation were seen in which the inner wall grew out of the sheath, behaved as a hypha of limited growth, and 'formed chlamydospores in the usual way'.

Macro- and micro-conidial formation has also been studied in *Sphaeronaema fimbriatum*. Halstead and Fairchild (1891) find that the tip of the

[*Annals of Botany, N.S. Vol. III, No. 12, October, 1939.*]

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conidiophore wall is ruptured, a microconidium is formed by a transverse septum, and is forced out by continued growth from below. Macroconidial formation differs only in size and rapidity. Lehman (1918) finds that the apical end of the protoplast of the conidiophore invests itself in a wall, and the microconidium so formed is liberated by the dissolution of the tip of the conidiophore wall: a second conidium may be formed before dehiscence, but those formed later are not endoconidia, as they are formed within an open sheath. In macroconidial formation the tip is dissolved away, the protoplast, surrounded by a delicate membrane, protrudes through the orifice, and spores are formed by the swelling and septation of the terminal portion. Successive spores are formed in the same way. Andrus and Harter (1933) agree generally with this description, but speak of 'intercalary elements' between the microconidia, and state that the first formed macroconidium is double walled.

Van Beyma (1935) has given a detailed account of the Thielavia stage of *Ophiostoma majus*. He states that spore formation agrees with that described by Brierley for *T. paradoxa*. In microconidial formation a terminal cell is cut off and the wall splits into two layers. The inner forms the spore wall, the outer a sheath through which the spores are extruded. Continued growth and septation of the protoplast and inner wall produces a chain of spores. Macroconidia are formed in the same way and may be ejected before maturity or remain within the outer wall until mature.

MATERIAL

The fungus used for this investigation was isolated from beech sawdust collected in a Bristol timber-yard during 1936. It was identified as *Ophiostoma majus* (van Beyma) Goidanich by comparison with type cultures from the Centralbureau voor Schimmelcultures, Baarn, Holland. It had been growing on 2 per cent. malt extract agar for two years at the time of investigation.

METHODS OF STUDY

Spore formation in *Ophiostoma majus* was studied in agar media in Petri-dishes, and in liquid media in hanging-drop cultures in van Teighem cells. The following media were used: 2 per cent. malt extract agar, 2 per cent. malt extract agar plus 1 per cent. N HCl, 6 per cent. oatmeal agar, Czapeks agar, and prune agar. The prune agar was made up as follows: 10 gm. dried prunes per 100 c.c. water were boiled for one hour in water, filtered, and the filtrate diluted to a specific gravity of 2.5. Two grammes agar were added per 100 c.c. of medium. The liquid media used were 2 per cent. malt extract, prune extract, made up as above without agar, tap-water, and distilled water.

The general course of spore formation was studied in living material, the rapidity of formation making it possible to follow the formation of a single spore throughout its life-history. Wall formation was studied in stained preparations. The very loose connexion between the young conidia and

conidiophores caused some difficulty in these preparations, and the majority were mounted in glycerine jelly to avoid undue displacement of the conidial chains by successive changes of alcohol. The stains used were safranin, gentian violet, erythrosin, and light green. Of these gentian violet was the most satisfactory, giving a particularly brilliant stain to delicate cell-walls. Plasmolysis of the protoplast of the conidiophore with 10 per cent. NaCl or 50 per cent. alcohol made the cell-walls more clearly visible in some cases. In unplasmolysed preparations the protoplast is in close contact with the walls of the conidiophore, and the structures are much obscured. After plasmolysis the wall layers and protoplast often become separated, and the various layers can be identified clearly. By the study of old and stale cultures it is possible to distinguish characters which are not visible in younger and more vigorously growing cultures.

DESCRIPTION OF THE CONIDIA

Conidia appear in a few hours in hanging-drop cultures of nutrient media grown at 27° C., and within two days at room temperature. On agar media they are produced abundantly in two days at 27° C., and after 5–7 days' growth at room temperature.

They are not produced until the mycelium has become well established, with considerable branching and septation. The more rapid production at high temperatures is due to the mycelium attaining this stage more rapidly, and is not due to any specific effect on the process of formation.

The conidia vary greatly in size and colour. In young and vigorously growing cultures brown or olive-green conidia are formed. These are oval, with a hyaline wall through which the contents of the conidium can be seen. Highly refractive aggregations of granules are characteristic of this form. Usually two such aggregations are formed, one at each end of the spore, but spores containing a number of such masses at various positions in the cell, a single mass, or not containing any, are occasionally found (Pl. XXIV, Fig. 1). The spore walls show characteristic lines of protuberances running in lines from end to end of the spore (Pl. XXIV, Fig. 7, older conidia in the chain). The spores are formed in chains containing up to twenty-five spores, and measure 7 μ–16 μ × 7 μ–11 μ, averaging 11 μ × 6 μ.

In older cultures spherical or pear-shaped spores, black or very dark brown in colour, with an opaque cell-wall, are formed. They are formed in short chains of 2–6 spores, either at the base of chains of the hyaline type (Pl. XXIV, Fig. 1) or on independent conidiophores (Pl. XXIV, Fig. 4). They measure 14 μ–22 μ in diameter, average 18 μ. They are formed typically only in old and stale cultures, after the formation of the hyaline type. In media with little food content, e.g. tap-water, no hyaline spores are formed, the opaque spores being formed shortly after germination.

A large number of forms intermediate between the hyaline and opaque

forms are found in old cultures. Conidial chains in such cultures frequently show transition in form from apex to base (Pl. XXIV, Fig. 7).

METHOD OF FORMATION

The conidiophore.

A short lateral branch arises from a vegetative hypha. This conidiophore may arise from any cell and does not appear from any particular part of the cell. Occasionally it is formed by the modification of the tip of a vegetative hypha. In the early stages it cannot be distinguished from a vegetative hypha. Later in development, after spore formation has commenced, the lower portion divides to form 1-4 short cells, and the apical, conidium-forming cell develops a typical slightly flask-shaped outline (Pl. XXIV, Figs. 1, 2, 7).

The conidia.

The first sign of spore formation is a slight swelling of the tip of the conidiophore. The tip of the outer wall is dissolved away, and the protoplast of the cell, surrounded by a delicate inner wall, is extruded from the opening. It has not been possible to identify this delicate inner wall before the extrusion of the first-formed spore. On plasmolysis the inner wall remains in contact with the outer wall of the conidiophore, and in some preparations can be distinguished for some distance below the mouth. It is very much thinner than the outer wall, and exact measurements are not practicable. As is pointed out by Lehman (1918), the even edge of the mouth of the outer wall indicates that it is formed by the dissolution of the tip, and not by rupture (Pl. XXIV, Figs. 1, 10). The protoplast within the extruded portion of the inner wall separates from the protoplast of the terminal cell. The two protoplasts remain in close contact until spore formation is complete, the line of separation being visible as a slightly hyaline area in the living cell. In some plasmolysed preparations the two become widely separated (Pl. XXIV, Fig. 8). The separation line is typically formed at the level, or slightly above the level, of mouth of the outer wall. More rarely it is formed within the outer wall (Pl. XXIV, Fig. 9).

The separated protoplast of the tip rounds off to form the conidium and becomes invested in a thickened spore wall. The swelling of the protoplast during spore formation causes a dilatation of the inner wall of the conidiophore, and over the greater part of the surface of the spore the two walls become too closely in contact to be distinguished apart from each other. At the ends of the spores the inner wall of the conidiophore can occasionally be identified as a delicate wall running from spore to spore (Pl. XXIV, Figs. 1, 3, 4, 5, 6, 9, 10).

It should be noted that the spore walls are formed endogenously, and that the inner wall of the conidiophore takes no direct part in their formation. In older cultures the protoplast swells to a greater extent in the formation of the spores, and the spore wall becomes more strongly and irregularly

thickened, forming the spherical spores described above. The shape of the spore depends on the time at which the spore wall becomes rigid. If this takes place at an early stage, the lower part of the spore retains the shape of the outer wall of the conidiophore, giving a pear-shaped outline (Pl. XXIV, Figs. 6, 9).

In some cases in which the separation of the protoplast takes place while within the outer wall of the conidiophore the spore may develop a rigid wall before extrusion. Such spores are often found at the base of rounded and pear-shaped ones, and are apparently only formed when the conidiophore is ceasing spore production (Pl. XXIV, Fig. 3).

In no case is a second spore formed within the outer wall of the conidiophore before the extrusion of the previously formed spore.

In older cultures the inner wall of the conidiophore becomes more evident, owing to a slight pigmentation, and a shrinkage of the spores. It can be seen clearly that there are no transverse septa associated with the inner wall. The spores are formed by fission of the protoplast only, followed by the formation of a spore wall inside the inner wall of the conidiophore (Pl. XXIV, Fig. 3a).

DISCUSSION

Van Beyma (1935) describes macroconidia which appear in all stages of transition from hyaline, brown or greenish spores, with granular content, $10\ \mu \times 7.3\ \mu$ — $9\ \mu$ in size, to opaque brown or black spores, with rough spore wall, rounded or pear-shaped, $22\ \mu$ — $30\ \mu$ in diameter. Lines of protuberances on the wall of the smaller macroconidia run in 'polar rows'. These are clearly the spores which are described in this paper. He also describes small hyaline microconidia, average size $12\ \mu \times 8\ \mu$, which are not formed by the fungus in the cultures under observation.

The occurrence of strains of *Thielavia basicola* which form macroconidia, but no microconidia in culture, has been observed by Johnson and Valleau (1935).

It is agreed that the various forms of macroconidium of this species are formed in the same manner, the only differences being the time of formation and thickness of the spore wall. The thick-walled spores are formed by the fungus under adverse conditions.

Berkeley and Broome (1850), Zopf (1876), Gilbert (1909), are of the opinion that the macroconidia are formed as chlamydospores by the direct septation of a conidial branch. This view is still held by some workers (Brooks, 1929). The mode of formation in this species shows a much closer relationship to microconidial formation, confirming the views of Halstead and Fairchild (1891), Duggar (1909), and von Höhnel (1904). It is shown, however, that the method of formation differs in several important respects from the descriptions of macroconidial and microconidial formation given by all previous investigators.

The descriptions of macroconidial and microconidial formation given by Brierley (1915) for *Thielavia* and Lehman (1918) for *Sphaeronema* are the most similar to the method described for this species.

They agree in stating that the macroconidia are formed after extrusion from the outer wall of the conidiophore. Brierley, however, finds this kind of formation only occasionally, under exceptional circumstances. The inner wall is stated by Brierley to be formed by the fission of the wall of the conidium, and to be half the thickness of the lower part of the conidiophore wall.

Lehman describes a much thinner inner 'membrane'. He does not describe its origin, but his drawing shows it to be in close contact with the protoplast within the mouth of the outer wall.

In *Ophiostoma majus* the inner wall is similar in thickness to the inner membrane described by Lehman, but is not so closely applied to the protoplast within the outer wall. Its close contact with the outer wall after plasmolysis, and the apparent continuity of the two in the lower part of the apical cell, suggests that it is formed by some form of division of the conidiophore wall. The great delicacy of the inner wall has prevented any direct evidence of fission being seen.

The descriptions of both these workers agree in stating that the macroconidia are not formed endogenously, but by the exogenous septation of the inner wall or membrane. Brierley relates the process to normal chlamydospore formation, stating that the transverse walls are laid down simultaneously. Lehman states that in *Sphaeronema* the walls are formed successively.

In *Ophiostoma majus* the spores are formed successively, and the spores in a conidial chain often show a distinct gradation in maturity from conidiophore to apex.

The appearance of the first-formed macroconidium within a 'double' wall, described by Andrus and Harter (1933) for *Sphaeronema*, is similar in some respects to the appearance of the first-formed macroconidium of *Ophiostoma majus*. Their drawing (Fig. 1a, p. 1061) shows an outer wall of equal thickness to the wall of the conidiophore, of which it is a direct prolongation. It is considerably thicker than the inner wall of the conidiophore which surrounds the first-formed macroconidium in *Ophiostoma majus*. It is not clear how the conidiophore wall of *Sphaeronema* divides to form a complete outer spore wall around the first-formed conidium and an open tube from which the successive single walled conidia are formed. The formation which they describe indicates that the spore wall is formed by protoplasmic activity, and not by longitudinal fission of the conidiophore wall. They have not observed any wall surrounding the macroconidial chain as a whole. The 'intercalary elements' found between the newly formed microconidia show much similarity to the appearance of the inner wall surrounding a young living macroconidial chain.

The method of spore formation in this species differs from all previous descriptions in that they are formed endogenously, within the inner wall of

the conidiophore, which remains as a delicate sheath around the spore chain until after they reach maturity.

Van Beyma (1935) has described mature macroconidia enclosed within a delicate sheath, but draws incorrect conclusions from his observations. He is of the opinion that the sheath is the outer wall of the conidiophore, and uses this theory as evidence in support of his view that the macroconidia are mature forms of the microconidia. In the living material this impression is often given by a superficial examination, but by staining and plasmolysis the mouth of the outer wall of the conidiophore can be seen at the base of the chain. It is therefore shown that the sheath is actually the inner wall. He is apparently of the opinion that the presence of the sheath around the spore chains is a characteristic which is found only occasionally, around a few of the chains in old cultures. He has failed to observe the more delicate sheath around the hyaline macroconidia in young cultures, which is rarely visible in unstained preparations.

CONCLUSION

The macroconidia of *Ophiostoma majus* are endospores, formed by free cell division within the terminal cell of the conidiophore. The mode of formation shows many points of similarity to the mode of formation of the macroconidia in Thielavia as described by Brierley, and Sphaerонema as described by Lehman. The differences are visible on the plasmolysis and staining of the material. The macroconidia are not formed in the manner described by van Beyma.

SUMMARY

An account is given of the macroconidia of *Ophiostoma majus*. It is agreed that they are of the Thielavia form, and that the variations in form seen in culture are modifications due to variation in the external conditions, and to the age of the culture.

They are formed by the dissolution of the tip of the outer wall of the terminal cell of the conidiophore, and the extrusion of the protoplast, enclosed within a delicate inner wall. The protoplast in the extruded portion of the inner wall rounds off to form the spore, and is invested in a thick cell wall. Further conidia are formed in the same manner. The conidia are therefore endogenous.

The inner wall of the conidiophore remains as a sheath around all the macroconidia in a conidial chain, and becomes more easily visible in older cultures.

ACKNOWLEDGEMENTS

The writer wishes to thank Dr. A. H. Campbell and the staff of the Botany Department of the University of Bristol for much assistance in this investigation, and the Colston Research Society for a grant for the purchase of equipment.

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EXPLANATION OF PLATE XXIV.

Illustrating Mr. S. A. Hutchinson's paper on 'Macroconidial Formation in *Ophiostoma majus* (van Beyma) Goedanich'.

Fig. 1. Chain of conidia from culture aged six days, showing mature conidiophore, transition in spore form, refractive aggregations in the older spores, and the inner wall of the conidiophore surrounding the chain.

Fig. 2. Conidiophore from culture aged two days, forming oval spores with refractive aggregations outside the mouth of the outer wall.

Fig. 3. Chain of thick-walled spores from culture aged twenty days, showing partial retention of the youngest spores within the outer wall of the conidiophore, and the presence of the inner wall. 3a. Focused to show inner wall. 3b. Focused to show youngest spore.

Figs. 4, 5, 6. Thick-walled spores showing inner wall at their ends.

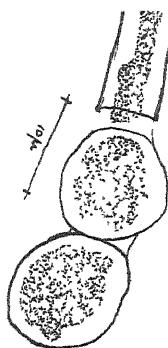
Fig. 7. Short chain of conidia showing transition in spore form.

Fig. 8. Single hyaline conidium from base of chain, showing mouth of outer wall below spore, and the ruptured ends of the inner wall above.

Fig. 9. Three hyaline conidia from base of chain, showing the inner wall within the outer wall of the conidiophore, and at the ends of the spores. The protoplast is dividing within the outer wall to form the youngest spore.

Fig. 10. Chain of two hyaline spores, showing the inner and outer walls of the conidiophore.

10

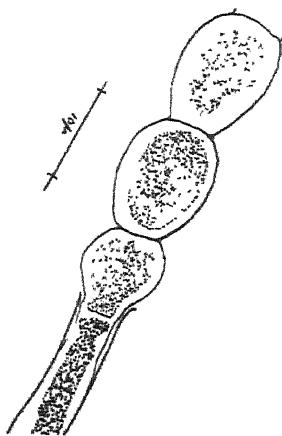


HUTCHINSON — MACROCONIDIA OF OPHIOSOTOMA.

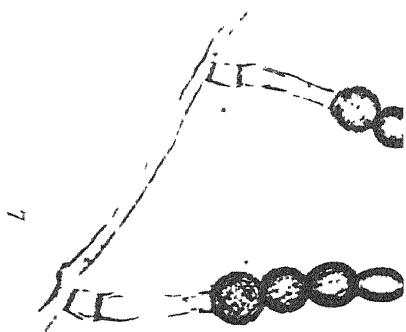
8



9



Huth, Stubbs & Kent.



7

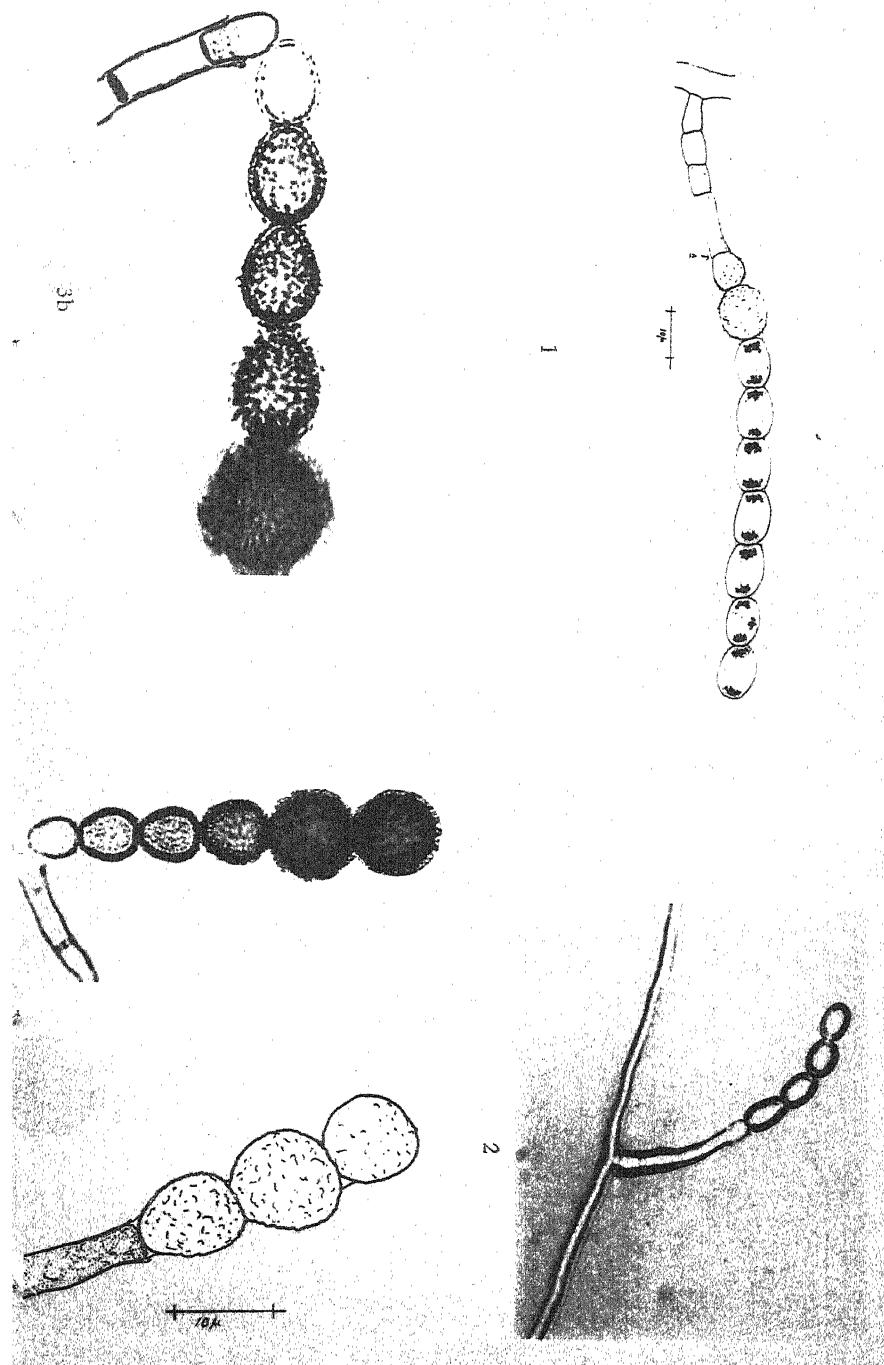


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6





The Effect of Prolonged Chilling on Water Movement and Radial Growth in Trees

BY

W. R. C. HANDLEY

INTRODUCTION

THE controversial subjects of water and food movements in plants have been approached from many angles. One experimental method has been to chill certain regions of the plant and to observe the effect upon the supply of water or metabolites to or from the region distal to the part chilled. As will be seen, however, from the literature discussed, the results of different workers have been too contradictory for any safe conclusions to be drawn on the subject of water movement.

Curtis (1929), using *Phaseolus vulgaris*, observed that chilling between 1° and 4° to 6° C. interfered with organic and inorganic solute transfer from roots to leaves, and that this abrupt check on translocation at temperatures around 1° C. to 5° C. coincided with the cessation of protoplasmic streaming. But at the same time, he concluded that such temperatures did not interfere with water movement; he stated that although on several occasions the external conditions were such as to cause high rates of transpiration, and sometimes even slight wilting, there was no difference between the turgor of the leaves of the control plants and those with chilled petioles, nor did plants with the main stem chilled differ from the controls in this respect. Zijlstra (1910) also found that cooling had no effect on water movement in *Helianthus*, whilst Polunin (1933) stated that the roots of *Betula odorata* conduct water at 0° C.

On the other hand, Kosaroff (1897) found that when lengths of 0·5 to 2·0 metres of the stems of *Phaseolus multiflorus*, *Humulus Lupulus*, *Lonicera sempervirens*, and *Passiflora coerulea* were cooled to 0° C. to -2° C., the leaves above the cooled region began to droop in two to four hours, and plants of the first two species died subsequently. On the other hand, no drooping was produced in plants of *Ampelopsis*, *Aristolochia*, and *Vitis* after cooling the stems at -4° C. or -5° C. for two to three hours, though young shoots of *Ampelopsis* and *Wistaria* drooped and died after cooling to -1·5° C. Also, Ursprung (1906) cooled 14 cm. regions of beech branches 60 to 80 cm. long with ice, and found that the leaves shrivelled in two to seven days. The same result followed when a similar region of a branch was killed by induction currents, heat or ether.

The present experiments were carried out to investigate the somewhat

divergent results of Curtis and Zijlstra as compared with those of Kosaroff and Ursprung, with regard to the effect of cooling on water movement, and also to determine the effect of the checked translocation on extension and radial growth of trees. It was also considered possible that such experiments might affect bud-scale formation; if bud scales are formed as the result of a scarcity of metabolites at the vegetative apex, as seems possible by their formation at a time when the leaves are expanding and growing rapidly (Priestley and Scott, 1935), then chilling the stems and checking the downward translocation from the leaves (as claimed by Curtis) might lead to the formation of leaves rather than scales.

EXPERIMENTAL PROCEDURE AND RESULTS

Most of the previous chilling experiments, discussed above, lasted only a few hours, except those of Ursprung which extended over two to seven days, but the present experiments were of much longer duration, being continued from March 18 to August 18.

On January 30, 1936, two similar young saplings of *Acer Pseudo-platanus* L. and two similar saplings of *Fraxinus excelsior* L. were planted in soil in large pots and the pots were placed in moist sand. The plants, both experimental and control, remained in the open throughout the experiment. The chilling system consisted of $\frac{1}{4}$ -in. bore, thin-walled lead tubing, which was coiled as closely as possible over the entire main stem, and through this a 50 per cent. glycerol-water mixture, cooled in an Electrolux refrigerating system, was pumped continuously. In this way the temperature of the stem was maintained between 2° C. and 6° C., whilst lower temperatures down to -1° C. and -2° C. could be obtained when desired. The lead tubes were surrounded with asbestos paper and wood wool, followed by another system of lead tubing through which tap-water was kept flowing continuously. The whole system was further enclosed by a layer of wood wool and $\frac{1}{2}$ -in. thick felt, and finally a layer of waterproof paper. This system enclosed the entire stem, except for the terminal bud and the lateral branches. Chilling began on March 18. The growth record of the chilled trees may be summarized as follows:

Date.	Fraxinus.	Control Fraxinus.
March 24	No growth.	No growth.
May 1	No growth.	Buds swelling.
May 15	Terminal buds on lower branches swelling, main terminal bud healthy but not swelling.	Growing well.
June 29	Terminal bud formed, extension growth extremely small.	Terminal bud formed.
August 18	About same number of leaves as in control but only half the size. Extension growths of laterals almost equal to those of control.	Only a small amount of extension growth ($\frac{1}{2}$ – 1) both of terminal and lateral shoots.

Date.	Acer.	Control Acer.
March 24	No growth.	Buds swelling.
May 1	Buds on lower laterals swelling, buds of upper laterals appear dry. Terminal bud alive but not swelling.	Growing well.
May 15	Leaves of buds of lateral branches unfolding, terminal bud swelling slowly.	Growing well.
June 29	Rapid extension growth of terminal shoot and large uncooled lateral, about 9 in. in each case.	Extension growth (2 to 3") ceased, terminal bud formed
August 18 (experiment concluded)	More but slightly smaller and paler leaves than control. Uncooled lateral underwent a small amount of secondary extension growth to 10 in. Terminal buds formed.	No lammas growth on any of the shoots.

ANATOMICAL OBSERVATIONS

On August 18 the chilled trees and their controls were preserved for subsequent anatomical study, which gave the following result.

Chilled Fraxinus excelsior.

In the terminal extension shoot (1936 growth) the depth of xylem tissue produced from pith to cambium was about $150\ \mu$, and this tissue was lignified to within four or five cells of the cambium. A small amount of starch was present throughout the pith and cortex and some in the lignified region of the xylem. No sclerenchyma was formed.

Compared with this, the 1936 extension growth of a lateral on the chilled tree produced xylem 150 – $300\ \mu$ in width, and this was lignified up to the cambium. At a few points on the periphery there was an indication that the cambium had had two periods of activity. Starch had accumulated at the periphery of the pith and in the inner regions of the cortex to a greater extent than in the terminal shoot, but there was relatively less in the central pith and outer cortex. Sclerenchyma was developed in isolated patches.

In the region enclosed by the cooling system, the first section was taken 3 in. below the terminal extension shoot. This showed a cambial region four or five cells wide, to the inside of which occasional new vessels had been formed on the periphery of the old wood, but the walls of these were unthickened and unlignified and the protoplasts of the segments were still present. Appreciable starch was present only at the periphery of the pith, none being present in the cambial region and little in the phloem. Half-way down the stem and at the base, the peripheral row of unthickened, unlignified vessels with their contained protoplasts could still be recognized; the diameter of these was only about half that of the 1935 early vessels, formed before the commencement of the experiment.

Control Fraxinus excelsior.

In the control the xylem tissue was about 200 – $300\ \mu$ wide and was lignified to the cambium; in structure this was similar to that in the chilled specimen.

Starch was very abundant around the periphery of the pith and in isolated cells in the more central pith, and was also present in wood and cortex. The sclerenchyma was formed as a continuous band.

Just below the terminal extension shoot, the vessels of the 1936 radial increment were larger than those of the 1935 wood (formed during extension), the new xylem was lignified up to the cambial region, which was four to five cells in width. Much starch was present in both xylem and phloem. Half-way down the tree, the 1936 increment was a normal, though narrow, wood ring; the vessels were rather smaller than in the 1935 increment and little late wood was to be recognized. The greater amount of starch in the phloem, as compared with the chilled tree, was very marked. At the base of the tree the structure was similar to that half way down, except that the late wood was more developed.

In *Fraxinus* the main differences between the chilled and control trees were the very small number of new vessels, relatively undifferentiated and still containing protoplasts, formed in the chilled tree, and the presence of little starch in the phloem and less in the wood than in the control tree.

Chilled Acer Pseudo-platanus.

In the chilled tree a section at the base of the extension shoot (1936) showed a xylem ring, the outer part of which was unlignified except for the rays, vessels, and cells immediately around the vessels, to a depth of four or five cells from the cambium; the cells, apart from those mentioned as lignified above, were also unthickened. Some starch was present at the periphery of the pith, but there was scarcely any in the phloem.

Half-way down the stem was slightly eccentric. A few small vessels with walls thickened and slightly lignified were present on the periphery of the 1935 wood on the larger radius of the stem, but on the smaller radius none had been formed. Starch was absent from the phloem and little was present in the xylem. Immediately below a large lateral near the base of the tree, the stem was markedly eccentric, the larger radius being below the lateral branch. The new vessels were present as a single row on the larger radius and decreased in number towards the smaller radius. Very little starch was present in the phloem, but was present in quantity right up to the cambium in that sector of the xylem containing the more numerous vessels, i.e. below the lateral branch; elsewhere much less starch was present, especially near the cambium. At the base of the stem the structure was similar to that immediately below the lateral branch, though the vessels were still more markedly restricted to the larger radius. Here the new vessels were smaller than those of previous rings, some had their walls only slightly thickened and lignified and still contained protoplasts.

Control Acer Pseudo-platanus.

In the terminal extension shoot the xylem ring was about the same width as in the chilled tree, but was lignified up to the cambium. Much starch was

present at the periphery of the pith and in the rays and parenchyma around the vessels, extending nearer to the cambium than in the chilled tree, and some was also present in the phloem.

Half-way down the stem, the xylem elements of the 1936 increment were thickened but unlignified. The ring was wider than that formed in 1935. Starch was present in moderate amount in the xylem, except near the cambium, and some was present in the phloem. At the base of the stem the 1936 increment was about the same width as that of previous years; the last-formed elements were thickened and had begun to lignify. Starch was present in quantity in the xylem, except to within about twelve cells of the cambium, and an appreciable amount was also present in the phloem, especially in the part more distant from the cambium.

In *Acer*, as in *Fraxinus*, the chilled tree showed an effect on vessel development and a smaller amount of starch, especially in the phloem.

Thus in both these species of trees the chief effects of the chilling on radial growth and translocation seemed to be that radial growth had been kept almost at a standstill; also, movement of starch from the leaves had apparently been impeded. Even where the influence of the strong lateral shoot on the chilled *Acer* exerted an effect on the main shoot in spite of the chilling, extremely few vessels were formed.

In both species normal terminal buds with bud scales were formed. The large lateral shoot of the *Acer* underwent some secondary growth, but this was a common phenomenon in *Acers* during this season, so that no significance can be attached to it in the experimental tree.

The most important result of the above chilling experiment came from a slight over-cooling. On June 12 at 9 a.m. the refrigerator was adjusted to full cooling power as the outdoor temperature was rather high and it was feared that the temperature of the chilled trees might rise above 6° C. At 6 p.m. on June 12 the leaves of the chilled trees were wilted, although wet due to slight rain; there was ice on the cooling tubes around the distal end of the 1935 extension growth, the air temperature between the cooling tubes and the shoot being just above 0° C. The roots of all the plants were well supplied with water and the leaves of the control trees were quite turgid. The refrigerating system was now adjusted so as not to chill so strongly, with the result that at 9 a.m. on June 13 the leaves of both the chilled trees had quite recovered and were fully turgid.

The above observation was repeated on June 15. At 9 a.m. the temperature of the air around the stems of the chilled trees was *Fraxinus* 4° C., *Acer* 2° C., the leaves of both being quite fresh and turgid. The refrigerator was then adjusted to give strongest cooling. At 3 p.m. on June 15 the temperature of the chilled trees was *Fraxinus* $\frac{1}{2}$ ° C., *Acer* $\frac{1}{4}$ ° C., the *Fraxinus* was quite fresh, and the *Acer* slightly wilted (especially the large lateral shoot). At 6 p.m. on June 15 both the chilled *Fraxinus* and the *Acer* were wilted. On June 16 at 9 a.m. the chilled *Fraxinus* and *Acer* were very badly wilted, the

temperature being -1°C . The refrigerator was then adjusted to the slowest rate of cooling, and although at 1 p.m. there was still a little ice on the cooling tubes, the leaves of the terminal shoot of the chilled *Acer* had recovered, those of the lateral shoots being only half recovered. The recovery of the leaves of the chilled *Fraxinus*, if any, was imperceptible. At 6 p.m. on June 16 the *Acer* tree had completely recovered (temperature 2°C .), the *Fraxinus* tree being half recovered (temperature 3°C .). On June 17 at 9 a.m. the chilled *Fraxinus* and *Acer* had both completely recovered, the temperatures now being *Fraxinus* 4°C ., *Acer* 2°C .

These experiments appear to show that chilling the stem from about 2°C . to about 0°C . cuts down the flow of water to the leaves to an almost negligible amount. Further, it tends to explain the discrepancy in the observations of Curtis and Zijlstra and those of Kosaroff and Ursprung, in that from the above experiments it seems that Curtis and Zijlstra did not use temperatures sufficiently low (for the particular species they used) to arrest water conduction, and apparently around the critical temperature two degrees has a relatively large effect.

The above experiment was repeated on several subsequent occasions, always giving the same results, except on August 12, when the wilting was allowed to go on too long and as a result some of the leaves died and dried out.

The fact that trees could produce more leaves and extension growth (at any rate in the case of *Acer*) than the control and maintain the leaves in a state of turgidity for months at a temperature of about 2°C ., but on chilling to 0°C . or a little lower they wilted very badly, seems to be very important in relation to problems of the ascent of sap in trees.

DISCUSSION

The theories of the mechanism of the ascent of sap in trees may be divided into two groups—the physical and the physiological or vital theories.

The physical theories purport to show that the upward movement of water in trees is due solely to physical phenomena, such as Jamin chains, imbibition of water by the cell-walls followed by movement of water along the walls, the movement of water as water vapour or as unbroken columns of water. Most of these processes have at one time or another been shown to be quantitatively inadequate, at any rate from water requirements as measured by the absorption of water by cut stems, or the rate of conduction of water by isolated pieces of wood; but such measurements, and even the more recent ones of Huber (1935) using thermo-electric methods, give no idea of the transpirational requirements of undisturbed trees.

After condemning other physical theories, Dixon and Joly (1895) and Askenasy (1895) simultaneously put forward the 'cohesion theory', in which the water is pulled up the xylem vessels of the stem in a continuous column by the transpiration of the leaves. Such a mechanism requires the existence of

high tensions in the vessels, and also that water should ascend through a dead portion of a stem to a living region beyond for an indefinite period, as was pointed out by Jost (1905). This requirement has never been demonstrated, as the shoot above the killed region never survives more than a day or two, often much less; Dixon (1914) explains this as due to the poisoning of the leaves by the substances produced by the dead cells of the killed region, and in other cases to blocking of the vessels by gum, &c., on killing.

Ursprung (1905*a* and *b*, 1906, 1907*a* and *b* and *c*) used stems killed with steam to demonstrate the necessity for living cells in the ascent of sap, and had much controversial argument with Dixon regarding the question of leaf poisoning. Roshardt (1910), who extended Ursprung's work, showed that apparently neither poisons nor displacement of the contents of vessels nor stoppage of the vessels occurred as a result of killing the stem with steam, and thought that the final decrease in water transport was due to a lack of energy which the dead cells could not remedy.

The results of the present experiments suggest that such drastic treatments as killing regions of stem or experimenting with shoots separated from the main tree are unnecessary in order to test the physical or vital theories of the ascent of sap. It is well known that around a critical point protoplasm is very sensitive to relatively small changes of temperature, which, although they leave physical phenomena unchanged, have considerable effect on protoplasmic activity.

In the present experiments, it seems probable that the process of chilling arrested the activity of the living cells of the wood merely temporarily. Also it should be noted that, whilst at 2° C. to 6° C. extension growth (except in the case of the terminal shoot of the chilled *Fraxinus*) and water conduction (as shown by turgidity of the leaves) proceeded quite normally, when, however, the temperature was lowered to 0° C., water conduction was almost, if not quite, stopped; but what is more important, the plants recovered on raising the temperature to 2° C. to 6° C. again and continued to grow and transpire as before, i.e. the cause of wilting was not death of the leaves.

In view of the fact that the trees were at the most 4 ft. in height and that the leaves and roots were out of the influence of the cold, it is impossible that such a small interval of temperature could in any way materially alter the previous physical condition of continuous water columns in the vessels, if indeed such are present and in any way connected with the ascent of sap. Hence on lowering the temperature of the stems the leaves should have continued to obtain water from the continuous water columns in the vessels as before, but the leaves wilted. The objection cannot be raised that the water in the vessels might be frozen, for according to Dixon (1914) freezing of the sap in the vessels only occurs at -11° C. and melting at -4° C. or -5° C., and Ewart (1908) stated the freezing-point of water in 0.7 mm. capillaries to be -7° C., whereas in the present case the temperature never went below -1° C. Further, the viscosity of the liquid in the vessels is not

materially altered by a change in temperature of two degrees, nor does it seem possible that it could have any significant effect on other physical processes such as imbibition.

Godlewski (1884) put forward a theory in which he postulated a pumping action of the ray cells combined with a decrease in air-pressure in the vessels with height in the tree. Janse (1887 and 1913) in general supported Godlewski and assumed that the wilting of a branch when the lower part is killed is due to interference with the vital sap-raising function of the parenchyma; he further conceived of a polarized pumping action of the ray cells.

Ursprung (1907*b*), Schulz (1882), Nordhausen (1921), Reinders (1910 and 1913), and Schwendener (1909) support the idea that ascent of sap is in part due to the vital forces of living cells.

Ewart (1906) and Peirce (1936) came to the conclusion that the parenchyma 'conditions' the water in the vessels and does not compel water movement, neither does it accomplish or effect the movement of water through the bodies of vascular plants. Peirce goes one step farther and states that water ascends the tree chiefly as water vapour, but this idea is not supported by the present experiments. On chilling to 0° C., the water vapour in the vessels would condense and cause wilting, but on recovery *Fraxinus* should recover more quickly than *Acer*. As the vessels of the former are longer, vapour movement will be restored more quickly when the temperature is raised as there are fewer separate vaporizing and condensing systems to set in motion again between the roots and the leaves, but in every instance, although its temperature was higher than that of *Acer*, *Fraxinus* took longer to recover.

Another postulated mechanism is that the differentiating xylem tissue draws water from the old wood and then propels it to the leaves in contact with the new xylem; this idea also seems inapplicable to the above experiments, for the unchilled differentiating xylem in the extension growth and the whole of the unchilled lateral shoot of the chilled *Acer* was unable to draw water from the old wood and supply it to the leaves when the temperature was lowered. The extra cooling from 2° C. to 0° C. or 1° C. may have broken the continuity of the extremely few living lines of new water-raising xylem by suppressing the activity of the very small amount of new xylem in the chilled region. This, however, does not seem to be the case, for in the 'snake' spruces and clipped hedge trees, the new xylem often does not reach the base of the tree for years at a time (Nordlinger, 1874), and in these cases the discontinuous new xylem functions efficiently if it is functioning at all.

Leclerc du Sablon (1910) thought that the principal factor in the ascent of sap is the osmotic pressure of the wood parenchyma cells which tends to keep the quantity of water constant, and also stated that transpiration accelerates the rate of flow but does not cause it.

Finally, there is the hypothesis of Westermaier (1883 and 1884), who considered that living cells alone were responsible for supplying sap to the

leaves. He considered the upward passage of water to be effected in the wood parenchyma whilst the vessels and tracheides acted as reservoirs rather than as conducting pipes.

Concerning the significance of the living cells of the wood in the problem of ascent of sap, *Carica Papaya* is an interesting plant which has extraordinarily few dead, lignified xylem elements, the bulk of the xylem being unlignified parenchyma. The stem lasts a number of years, attaining a height of 25 ft. and a diameter of 5 in., and the plant also has very large leaves. Considering the very small number of dead water channels in the xylem, it is difficult to see how the cohesion theory can apply in this case; if it does, the vessels must be remarkably efficient in transport even on a very moderate water requirement.

The chilling experiments described in this paper seem to indicate that living cells are involved in the ascent of sap. At temperatures around 0° C. and 30° C. (depending on the species) there is a sudden cessation of protoplasmic activity. Thus Curtis (1929) stated that the seemingly abrupt check in translocation at temperatures around 1° C. to 5° C. coincides approximately with complete stoppage of protoplasmic streaming. In plants such as *Nitella* and *Elodea* streaming does not completely cease until the temperature drops to 0° C. Coupling such facts with the present observation that an interval of 2–3° C., at a mean temperature about 0° C. (far above the demonstrated freezing-point of the liquid in the vessels), caused wilting, which could not be due to any physical change occurring in the liquid in the vessels, it would seem that the ascent of sap involves a chain of living cells continuous from roots to leaves.

This would appear to indicate that vessels and other dead xylem elements are the incidental results of the metabolic environment of the cambium at the time of their formation, and are unconnected with water availability or conduction, except in the role of reservoirs. If the living cells of the wood are active agents in the ascent of sap, the difficulties of others in explaining the ascent of sap in winter do not arise. The present chilling experiments have the advantage that they were carried out on entire living trees.

At the present time a theory of ascent of sap, entirely dependent upon a living system, is open to the criticism that it appears quantitatively impossible. On this point, however, the criticism cannot carry great weight until our knowledge of the water requirements of trees under natural conditions is more complete, and until greater knowledge is available of the rate at which water may move through a living cell.

SUMMARY

1. The woody shoots of young saplings of *Fraxinus excelsior* and *Acer Pseudo-platanus* in pots were subjected to continuous cooling to about 2° C. during the growth season, with the result that radial growth was almost completely inhibited throughout the woody stem.

2. The chilling did not adversely affect extension growth except that it was later in commencement and proceeded more slowly.
3. If the temperature around the stem is lowered from 2° C. to 0° C., water conduction is cut down to such an extent as to cause wilting of the leafy shoots; turgidity is recovered when the temperature is again raised to 2° C.
4. This wilting effect is discussed particularly in relation to the part played by living cells in the upward movement of water in the wood.

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Studies in the Proteaceae

III. Embryology of *Grevillea Banksii* R. Br.

BY

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With twenty Figures in the Text

INTRODUCTION

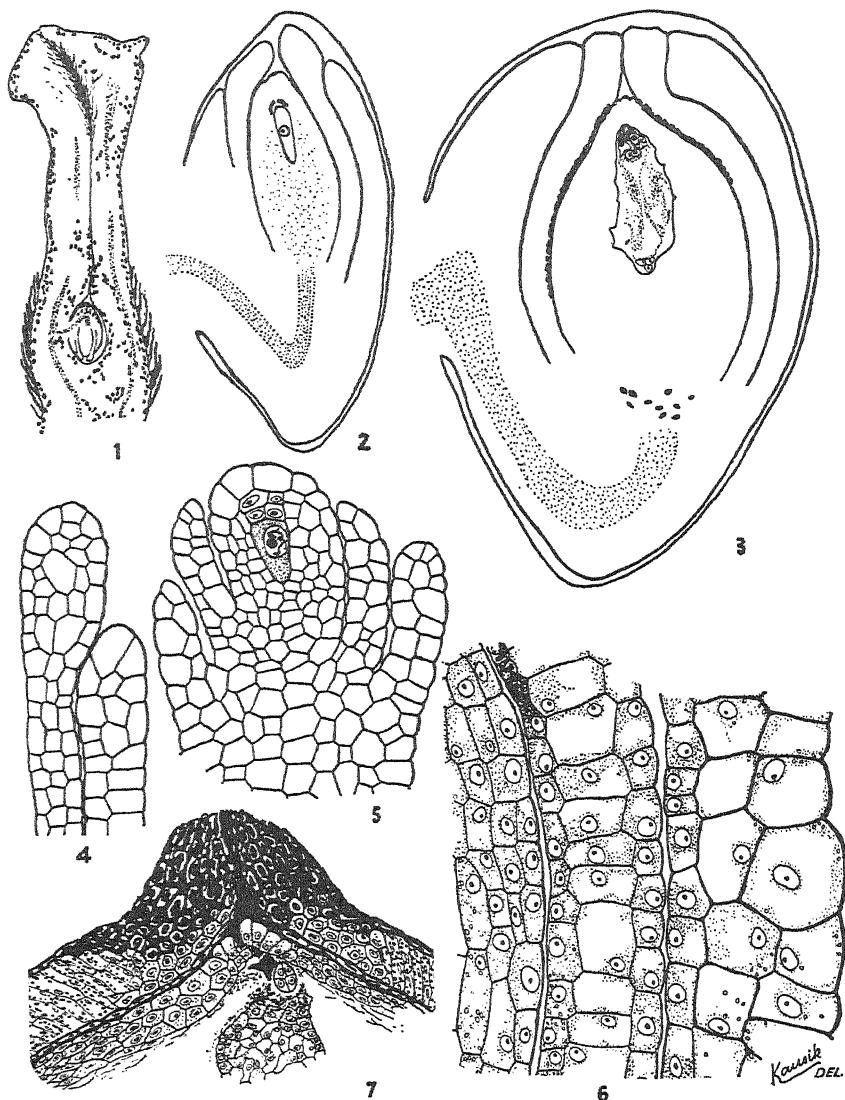
IN the following study an attempt is made to examine in detail the important features of the development of the embryo and endosperm in *Grevillea Banksii* R. Br. While in main the results are similar to those in *Grevillea robusta* (Kausik, 1938) and *Macadamia ternifolia* (Kausik, 1938a), a few interesting points, which are not included in the two previous communications or appear to be rather different, are specially dealt with here.

Grevillea Banksii R. Br. is a woody shrub, the flowers of which are fairly large and brick-red in colour forming dense inflorescences. The material on which this study is based was collected during the month of July 1938 from plants grown at the Government Botanic Gardens, Ootacamund, and at Sim's Park, Coonoor, Mount Nilgiris, South India. Bouin's fluid was as usual employed for fixing the material, and after the processes of dehydration and infiltration sections were cut from 8 to 14 μ . Heidenhain's iron-alum haematoxylin was used as a stain.

Considerable difficulty was encountered in sectioning the material, as the ovary, even in very young stages, is densely clothed externally with numerous pointed hairs. Removing these hairs at the time of fixing the material was found to be a laborious process as the young ovaries are too small to be handled conveniently. Further, attempts to shave away the hairs often result in injuring some portions of the ovary itself. The difficulties of sectioning were, however, later overcome by scraping the material when in xylol before paraffin infiltration; the hairs then come away easily as a felted mass.

THE OVULE

The ovary is made up of a single carpel and contains groups of darkly staining tannin cells in its tissues (Fig. 1). There is a long style terminating in a hard disc-like stigma which contains a pollen-collecting chamber shaped like a funnel. The inside of the chamber is lined by numerous glandular cells. Within the ovarian cavity are found two large ovules which are attached by thick and stout funiculi (Fig. 1). The ovules may be described as



Figs. 1-7. Fig. 1. Longitudinal section of a young ovary showing the ovule, vascular strands, and the hairs of the ovary wall. The large dark spots represent tannin cells. $\times 20$. Figs. 2 and 3. Longitudinal sections of young and old ovules in outline. The stippled nucellus in Fig. 2 indicates the presence of rich contents in the cells. In Fig. 3 groups of tannin cells at the region of the micropyle and chalaza are shown. $\times 120$. Fig. 4. A portion of the integuments from Fig. 2 enlarged. $\times 400$. Fig. 5. A very young ovule showing the two integuments, the megasporangium, and the four small parietal cells. $\times 400$. Fig. 6. A portion of the integuments and the periphery of the nucellus enlarged from Fig. 3; part of the tannin layer formed by the innermost layer of the inner integument is shown advancing towards the base. The small circles are grains of starch. $\times 600$. Fig. 7. The micropylar portion of the

amphitropous and are provided with two integuments with the micropyle pointing towards the base of the ovary. In the formation of the micropyle only the inner integument takes part. As will be described presently, the ovule, when fully developed, has a massive nucellus at the base of which develops a pad of tannin cells, the hypostase. The latter is a conspicuous feature of the developing seed.

A single archesporial cell becomes differentiated in the nucellus of the young ovule. This is hypodermal in origin and divides to form a parietal cell and a megasporangium. The former immediately divides twice by longitudinal and transverse walls to form a group of four parietal cells (Fig. 5); the megasporangium enlarges in size in the meanwhile. At this stage the two integuments are quite conspicuous; there is no reason to suppose that they grow slowly, as stated by Brough (1933), for, according to him, a characteristic feature of the ovule in *Grevillea robusta* is the slow growth of the integuments. This, however, is not in agreement with my results (Kausik, 1938).

Both the integuments are at first two cells thick, but as development of the ovule proceeds the inner integument becomes three-layered while the outer remains unchanged for some time (Fig. 4). Gradually, however, as the ovule becomes older, and at the time when it contains a fully developed embryo-sac, the outer integument also forms three layers of cells on the outer free side of the ovule and more than three layers on the inner funicular side (Fig. 6). In the inner integument, the cells forming the innermost and the outermost layers are small and have rich contents; the cells of the innermost layer also include grains of starch. As the ovule grows further these starch-filled cells form a layer of tannin cells. The development of the tannin layer first begins at the region of the micropyle and gradually extends towards the base of the ovule. (Figs. 3 and 6). The cells of the middle layer of the integument are large and vacuolate. They are radially elongated and develop innumerable starch grains as the seed is formed. The cells of this layer in the region of the micropyle also divide once tangentially to form two layers (Fig. 7).

As these changes are taking place in the inner integument with progressive development of the ovule, the outer integument also shows changes as growth proceeds. As already stated, it becomes three-layered sooner or later, of which the innermost layer alone is made up of small cells with rich contents. The cells of the outer two layers are large with thick walls, staining light brown with haematoxylin. These cells have a little cytoplasm, a few grains of starch, and large vacuoles (Fig. 6). As the ovule develops into the seed, the outer integument becomes very thick with a number of layers of regularly arranged cells formed as a result of repeated tangential divisions to form the wing for

seed shown in Fig. 20 enlarged to show details, the outer integument being excluded. The inner integument shows the dark, thick-walled cells of the innermost and outermost layers and the starch-filled cells of the middle layer. A four-celled embryo and portions of the nucellus and endosperm are seen. $\times 200$.

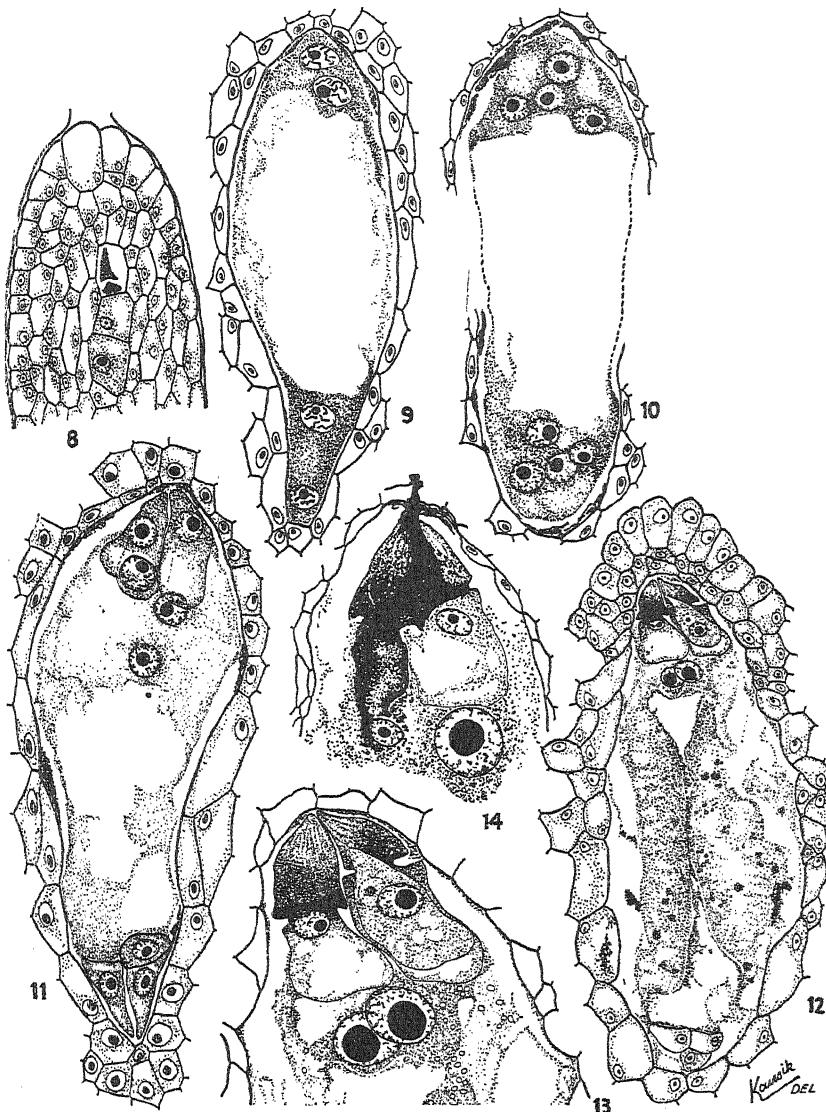
the seed (cf. Brough, 1933). The cells of these layers contain many starch grains but little else.

With the growth of the ovule the nucellus also becomes more and more massive. Several layers of nucellar cells are formed all round and below the embryo-sac. Most of these cells of the nucellus, except the peripheral two or three layers, contain starch grains. In *Protea Lepidocarpon* too '... the cells of the nucellus immediately surrounding the embryo-sac become densely packed with starch' according to Ballantine (1909). The upper portion of the nucellus above the micropylar end of the embryo-sac forms a conical protuberance and consists of glandular cells. The cells of this glandular apex are derived partly from divisions of the original four parietal cells and partly from the overlying epidermis. At the base of the ovule the nucellus forms an extensive mass of nutritive cells, below which is seen a zone of meristematic cells. The activity of these cells, particularly after fertilization, results in the formation of additional regularly arranged cells, which are completely destroyed by the encroaching endosperm later. The lowermost limit of the nucellus is marked by the region where groups of tannin cells arise (Fig. 3). These tannin cells become more extensive and form a pad, the hypostase, in the developing seed (Fig. 15). The hypostase is of common occurrence in the Onagraceae, and according to Johansen (1938) 'it is a flexible adaptation to the environment that makes its appearance only when necessary and serves to stabilize the water balance of the resting seed over the long period of dormancy during the hot dry season' (Capoor, 1937). The vascular strand of the ovule ends near the hypostase.

DEVELOPMENT OF THE EMBRYO-SAC

The megasporangium undergoes the usual two divisions to form the linear tetrad, the lowest megasporangium of which develops into the embryo-sac of the normal eight-nucleate type, while the upper three megasporangia degenerate (Fig. 8). At the four-nucleate stage of the embryo-sac the two nuclei at the antipodal end are arranged either side by side or one below the other. After the eight nuclei are formed (Fig. 10), the embryo-sac begins to organize itself and contains two sac-like synergids, the egg, two polar nuclei in the upper half of the embryo-sac, and three antipodal cells. Two of the last are pointed posteriorly and include one or two small vacuoles (Fig. 11). Further growth of the embryo-sac proceeds till the time of fertilization. During this period the synergids become more prominent and develop the filiform apparatus at the apex, separated from the basal vacuolate portion by a deep notch in the middle (Figs. 12 and 13). The egg cell lies in juxtaposition with one of the synergids and nearby are found the two large polar nuclei lying in close contact. The antipodals are less conspicuous now than at the stage when they were just formed (Fig. 11); they are nevertheless evident and persist even after fertilization.

The fully developed embryo-sac, when ready for fertilization, is at least



FIGS. 8-14. Fig. 8. Nucellus with the linear tetrad of megasporangia, of which the upper two have already disorganized. $\times 450$. Figs. 9 and 10. Four-nucleate and eight-nucleate embryo-sacs; Fig. 9, $\times 800$, Fig. 10, $\times 900$. Fig. 11. Embryo-sac beginning to be organized; note the two antipodal cells forming the posterior pointed ends. $\times 900$. Fig. 12. Embryo-sac which is ready for fertilization; note the irregular outline and the presence of starch grains inside the embryo-sac. The glandular apex of the nucellus lying above the embryo-sac is also shown. $\times 400$. Fig. 13. The micropylar portion of the embryo-sac enlarged from Fig. 12; the filiform apparatus is well shown. $\times 900$. Fig. 14. The pollen tube lying near the egg apparatus; one of the two synergids is quite intact. $\times 900$.

twice as large as it was at an earlier stage (Fig. 11) when its organization had just begun. It becomes very irregular in outline as it develops at the expense of the surrounding nucellar cells, which not only lose their rich contents but also become irregular and loosely arranged. Further, their cell walls are gradually dissolved and the grains of starch which they contain become included in the general cytoplasmic mass of the enlarging embryo-sac (Fig. 12).

FERTILIZATION

The pollen tube passes through the glandular apex of the nucellus and enters the embryo-sac, destroying one of the two synergids (Fig. 14). In the entry of the pollen tube into the embryo-sac, the filiform apparatus of the synergids seems to have a definite role by secreting some chemotactic substance. The pollen tube appears very dense and apparently contains highly staining material which is discharged into the embryo-sac when the tip of the tube bursts. The details regarding the liberation of the male nuclei and of syngamy and triple fusion could not therefore be studied.

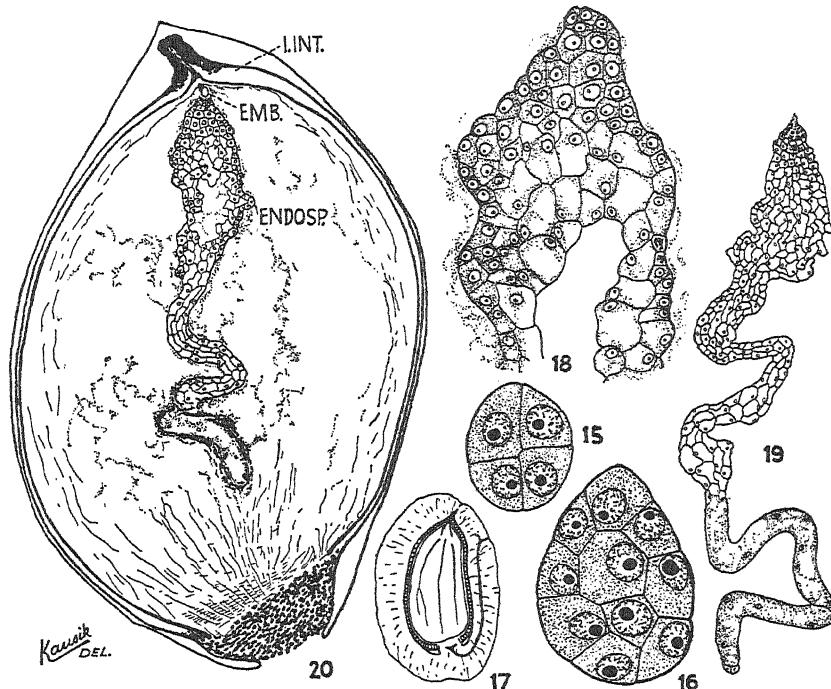
EMBRYO

The fertilized egg undergoes a long period of rest and divides only after a considerable amount of endosperm tissue is formed. The earliest stage of the embryo available for examination was four-celled with the endosperm as a large cellular mass (Figs. 15, 18, and 20). It may be inferred that the first division of the fertilized egg takes place by a transverse wall as in *Grevillea robusta* (Brough, 1933; Kausik, 1938) and *Macadamia ternifolia* (Kausik, 1938a). Gradually, as further development takes place, the embryo becomes many-celled. It is oval in shape and has a pointed proximal and a broad rounded distal end (Fig. 16). After the histogenic layers are differentiated, it forms the two cotyledons, the hypocotyl and the radicle. The fully developed embryo completely occupies the seed. The cotyledons are thin and expanded and as in the other members of the family studied previously, they are provided with basal lobes. The radicle is pointed and fits into the micropyle (Fig. 17).

ENDOSPERM

In the development of the endosperm free nuclei are at first formed, after which cell formation takes place. Cell organization is regular only in the upper half of the embryo-sac, being irregular lower down. As in *Grevillea robusta* (Kausik, 1938) and *Macadamia ternifolia* (Kausik, 1938a), here, too, distinct regions of the endosperm may be recognized. These are the upper, the middle, and the lower regions. The upper region of the endosperm contains regularly arranged compact cells with rich contents. Towards the inside of this region cell formation proceeds more or less irregularly, and is finally completely suppressed at the centre where no tissue is formed (Fig. 18).

The middle region of the endosperm is narrow and contains rows of elongated cells (Figs. 19 and 20). The lower region is completely devoid of cells but contains free nuclei scattered in a mass of highly vacuolate cytoplasm (Figs.



Figs. 15–20. Figs. 15 and 16. Four and many-celled embryos respectively. $\times 900$. Fig. 17. Longitudinal section of seed showing the wing formed by the outer integument, the inner integument and the embryo completely occupying the seed and showing the large cotyledon with its basal lobes and the pointed radicle. $\times 25$. Fig. 18. The upper portion of the endosperm from Fig. 20 enlarged to show the nature of cells. $\times 120$. Fig. 19. The endosperm, mounted whole, showing the upper and middle regions and the lower region forming the *vermiciform appendage*. $\times 40$. Fig. 20. Longitudinal section of seed, drawn from several sections, showing the endosperm lying loosely in the cavity formed by the destruction of the cells of the nucellus. The hypostase, the zone of meristematic cells in the chalaza, the inner integument, and the inner margin only of the outer integument are shown. $\times 40$.

19 and 20). This region is the tubular *vermiciform appendage*. Both this, and a part of the middle region of the endosperm, are thrown into a contorted form. The *vermiciform appendage* grows down towards the base of the ovule by destroying the cells in the chalazal region. In this connexion it is interesting to note the occurrence of a tubular embryo-sac in *Siparuna Eggersii* (Heilborn, 1931), which 'elongates considerably and penetrates downward into the nucellus but soon encounters a hypostase which checks further development. Here it becomes coiled into several turns and the end swells into a vesicle, which finally bursts and releases its contents into a nucellar

cavity that has formed in the meantime just above the hypostasis' (Maheshwari, 1937). The case of *Siparuna Eggersii* is, however, different, for fertilization does not occur and the posterior elongation of the embryo-sac to form the coiled tube cannot strictly be likened to the *vermiform appendage* of *Grevillea*, which is a haustorial organ formed by the endosperm after fertilization. The nature of the appendage is fully described in *Grevillea robusta* (Kausik, 1938), and in studying it here it was again found profitable to dissect out the seed and prepare whole mounts of endosperm as described in detail elsewhere (Kausik, 1938b).

During the development of the endosperm the cells of the nucellus all round the embryo-sac, and the nutritive cells formed by the meristematic cells at the chalazal region of the ovule, are completely broken down, a large cavity being formed inside the seed. The mass of endosperm is loosely placed within this cavity (Fig. 20). Later, when the seed becomes ripe, this cavity is occupied by the large embryo.

THE SEED

The seed is flat and non-endospermic when ripe. It has a broad wing which is formed by the outer integument. The wing is made up of several regularly arranged layers of cells. The inner integument remains three-layered. Its outermost and innermost layers show dark thick-walled cells which are elongated along the longitudinal axis of the seed, while the middle layer has radially elongated thin-walled cells containing starch grains. The inner integument forms a hard protective covering, though it is much thinner than the outer. A thin layer of nucellus persists inside the inner integument. The cavity of the seed is completely occupied by the large embryo. The chalazal region of the seed is constricted and the darkly staining hypostase is conspicuously seen here (Figs. 17 and 20).

CONCLUSIONS

As in *Grevillea robusta* and *Macadamia ternifolia* studied previously, the nucellus becomes more and more massive as the ovule develops and forms an extensive region storing much nutritive material. This material is made use of by the embryo-sac at different stages of its development. The cells of the chalazal region of the ovule, which are meristematic, divide actively and regularly, and give rise to additional layers of nutritive cells. These cells are formed especially after fertilization and provide an important nutritive source for the endosperm when it develops the haustorial *vermiform appendage*. A supply of nutritive materials is thus always available to the embryo-sac, both before and after fertilization.

The embryo-sac itself may be said to pass through two distinct phases before fertilization. The first of these covers all the stages that lead to the formation of the eight-nucleate embryo-sac. This is the formative period,

during which the cells immediately surrounding the embryo-sac are crushed and many of them lose to some extent their contents. The second phase of the embryo-sac begins from the time the eight nuclei proceed to organize themselves. This phase is marked by a great increase in the size of the embryo-sac accompanied by the destruction of more nucellar cells. These cells become much depleted of their contents and are found loose and irregularly arranged all round the embryo-sac. Even the walls of some of the cells are dissolved away and their cytoplasm and starch grains become merged in the cytoplasm of the embryo-sac. The result of these changes is the very irregular outline which the embryo-sac shows when it is ready for fertilization. The embryo-sac has now completed its second phase, the growth period, during which the organization of the egg apparatus, with the synergids forming the filiform apparatus, has taken place. The polar nuclei have in the meanwhile steadily increased in size and are lying closely pressed against each other and in the immediate neighbourhood of the egg apparatus.

The increase in the size of the embryo-sac does not stop at the end of the second phase, but is resumed again after fertilization when it becomes more pronounced. This is accompanied by the formation of the endosperm tissue, which develops more towards the chalazal region of the ovule to invade the large mass of nutritive cells. The embryo-sac thus becomes greatly elongated and narrow, with its posterior end forming a long tubular multinucleate structure which becomes twisted in its course. This is the *vermiform appendage* which is characterized by a haustorial role. The endosperm, when once it is formed, rapidly encroaches on all the nucellar cells and absorbs the materials contained in them. This feature is particularly noticeable near the chalazal region of the ovule, but along the sides some of the peripheral layers of nucellus situated farther away from the embryo-sac and in close contact with the integument do not appear to be accessible and persist even in the ripe seed. These layers appear as a crushed layer between the large embryo and the hard inner integument.

The formation of the endosperm and its appendage is essentially similar to that in *Grevillea robusta* (Kausik, 1938a). There is, however, a striking difference in that the narrow middle region with rows of elongated cells which is conspicuous here, is absent in *Grevillea robusta*. The intercalation of this region in *G. Banksii* indicates that cell formation, which is abruptly suppressed in *G. robusta* to form a sharply delimited *vermiform appendage*, is here more persistent. It is only gradually suppressed before the multinucleate tubular appendage becomes evident.

SUMMARY

The embryo-sac of *Grevillea Banksii* R. Br. develops according to the normal type. At the time of fertilization it has a very irregular outline. The synergids show a well-marked filiform apparatus.

The development of the embryo is similar to that in the two members of the family previously studied (Kausik, 1938, 1938a).

The endosperm becomes cellular. The presence of the *vermiform appendage* of the endosperm described in *Grevillea robusta* (Kausik, 1938), has been demonstrated here. Though similar in both, the endosperm of *G. Banksii* shows a narrow middle region with rows of elongated cells which are absent in *G. robusta*.

In conclusion, the writer has great pleasure in acknowledging his indebtedness to Prof. M. A. Sampathkumaran, of the University of Mysore, for encouragement and kind help during the course of this investigation. Thanks are also due to the Curator, Government Botanic Gardens, Ootacamund, and Sim's Park, Coonoor, Nilgiris, South India, for permission to collect the material for this study.

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Studies in Tropical Fruits

VI. A Preliminary Consideration of the Solubility of Gases in Relation to Respiration

BY

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With five Figures in the Text

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I. INTRODUCTION

IN the study of the respiration of plants a knowledge is required not only of the carbon dioxide liberated at the surface of the respiring organ and of the absorption of oxygen there, but also of the gaseous concentrations in the intercellular spaces of the tissues, of the amounts contained in the tissues at any time whether in solution or otherwise, and of the resistance offered to the movement of gases (Wardlaw, 1936; Wardlaw and Leonard, 1936, 1938).

Before carbon-dioxide production can be quantitatively related to changes in metabolites not only the amount of carbon dioxide liberated but also that held in the tissues and tissue spaces must be known. Preliminary determinations have shown that the quantity of carbon dioxide in the tissues of different tropical fruits differs considerably from that which would be present under similar conditions in an equal volume of water, that is, the gas appears to be retained under different conditions in biological and purely physical systems. As a contribution to this aspect of physiology it is proposed to

deal here with some preliminary considerations of the actual and relative amounts of the gases dissolving in water and various solutions under the several conditions of temperature, pressure, and gaseous concentration which may occur in plant tissues. Only the static quantities of gases in solution will be considered in this paper.

The biological significance of the relative solubility of the two gases chiefly involved in respiration has been the subject of a considerable literature. Henderson (1927), commenting on the physiological significance of the properties of carbon dioxide, says: 'were carbon dioxide not gaseous, its excretion would be the greatest of physiological tasks; were it not freely soluble, a host of the most universal existing physiological processes would be impossible.' In this paper, data are submitted which show that the solubility of oxygen in water, while very much lower than that of carbon dioxide, is likewise of vital importance. The solubilities of other gases, both those normally encountered in respiration studies and those known to produce physiological responses, also merit careful consideration along the lines suggested.

II. TERMINOLOGY

In the majority of biological phenomena the normal conditions involve consideration of the quantities of gases in solution in water and other liquids (i) in equilibrium with the partial pressures of nitrogen, oxygen, and carbon dioxide produced by concentrations of these gases of 79 per cent. for nitrogen and 21 per cent. for the sum of oxygen and carbon dioxide, (ii) at total pressures not widely departing from 760 mm. Hg (737–88 mm.), (iii) at temperatures ranging, for tropical fruits, from 60° to 120° F. (15°–40° C.). Solubilities at lower temperatures have, however, been embodied in the tables in this paper, since values for gas solubilities are generally given from 32° F. upwards.

The 'coefficient of solubility' (C) is the volume of gas at the given temperature absorbed by one volume of the liquid when the total pressure is 760 mm. Values of this quantity, or of other coefficients,¹ for different gases have been

¹ The Ostwald 'coefficient of solubility' (λ) of a gas is the ratio of the concentration of the gas in the liquid to the concentration in the gas phase.

$$\lambda = \frac{C_A}{C_A} = \frac{\text{concentration in liquid}}{\text{concentration as gas}},$$

and is independent of the partial pressure of the gas, according to the Henry-Dalton laws.

The Bunsen 'absorption coefficient' (α) is the volume of gas (reduced to N.T.P.) absorbed by one volume of the liquid at the given temperature when the pressure of the dry gas outside the liquid is 760 mm., that is, when the *partial pressure* of the gas is 760 mm.

$$\alpha = \frac{273 \cdot 1 \lambda}{T}.$$

For the coefficient of solubility (C) accordingly

$$C = \alpha \times \frac{760 - f}{760} \times \frac{T}{273},$$

where T = absolute temperature and f = vapour pressure of liquid at T° .

determined by various workers. They show considerable divergences due to experimental differences. The values used in this paper have been obtained from Bayley's 'Pocket Book for Chemists' (1937), or 'International Critical Tables', vol. iii (1928), or derived therefrom by use of the connecting formulae given in the footnote. The original publications, in most instances, have not been available.

The value C has been taken as giving the clearest representation of the amount of gas in solution under any given set of conditions, since it is probable that most biological gas phenomena involve the actual quantity of gas in solution in equilibrium with atmospheres saturated with water vapour. Even in the case of gaseous interchange at the outer surface of a fruit or other organ, a diffusion 'shell' of water vapour exists such that approximately saturation is maintained at the cell interfaces.

Throughout this paper the percentage concentration of the gases is used in preference to the partial pressure since it is the value readily ascertained by means of the Haldane Gas Analysis apparatus. The normal pressure used in the calculations is 760 mm., although the mean daily pressure in Trinidad, where the biological observations are being made, is slightly different (762 mm.).

Carbon dioxide is approximately thirty-five times as soluble as oxygen, which again is about twice as soluble as nitrogen at 0°C . The gases also differ in their temperature coefficients of solubility, the order being the same as that of their solubility.

In the sections which follow the tables of calculations have been given in some fullness as an illustration of the effects of these different solubilities and temperature coefficients of solubility and also as an introduction to the more complex relationships existing in biological systems involving gases and liquids, which are being made the subject of investigation.

III. THE SOLUBILITY OF AIR IN WATER IN RELATION TO TEMPERATURE

Taking as a simple example the case of normal air in equilibrium with air dissolved in water, the relevant figures for the constituent gases dissolved in 100 c.c. of water covering the range 0° – 35°C . and at a total pressure of 760 mm. Hg are shown in Table I. The composition of dry air is taken as nitrogen (and other inert gases) 79.04 per cent., oxygen 20.93 per cent., carbon dioxide 0.03 per cent. (Haldane, 1920).

It will be seen that for a temperature rise of 10°C . (25° to 35°C . = 77° to 95°F .) the volumes of the dissolved gases are decreased for nitrogen 11.55 per cent., oxygen 12.94 per cent., carbon dioxide 20.83 per cent. of the volume at the lower temperature, due to the different temperature coefficients of solubility. It will also be seen that, because of the different solubilities at any one temperature, whilst oxygen constitutes 20.93 per cent. of the *atmosphere*, the dissolved oxygen constitutes about 34 per cent. of the

TABLE I

Volume (c.c.) of the Constituent Gases of Air dissolving in 100 c.c. of Water (i.e. C_A) in Equilibrium with Normal Air at Different Temperatures

(Pressures are shown in mm. Hg.)

°C.	V.P. of water (Regnault) (760-f)	Nitrogen (79.04%)		Oxygen (20.93%)		Carbon dioxide (0.03%)	
		f	Partial pressure C _A	f	Partial pressure C _A	f	Partial pressure C _A
0	4.6	755.40	597.07	1.847	158.11	1.017	0.23 0.052
4	6.097	753.90	595.88	1.694	157.79	0.927	0.23 0.045
10	9.165	750.84	593.46	1.504	157.15	0.815	0.23 0.037
15	12.699	747.30	590.67	1.377	156.41	0.742	0.22 0.031
20	17.391	742.61	586.96	1.276	155.43	0.680	0.22 0.027
25	23.550	736.45	582.09	1.195	154.14	0.626	0.22 0.024
30	31.548	728.45	575.77	1.127	152.46	0.581	0.22 0.021
35	41.830	718.17	567.64	1.057	150.31	0.545	0.22 0.019

total volume of *dissolved gas*. The percentages by volume of the three gases of the dissolved air at different temperatures are given in Table II. It is suggested that it is the absolute amount of the individual dissolved gas at any time which is important in the biochemical processes in progress at that time: the *percentage* of the total volume of dissolved gases, on the other hand, may be important in the physical processes involved in gaseous interchanges.

TABLE II

Total Volume (c.c.) of Gases dissolving in 100 c.c. of Water in Equilibrium with Air at Different Temperatures and the Volumes of the Constituent Gases expressed as Percentages of this Total Volume

°C.	Total vol.	% of total volume of dissolved gas		
		N ₂	O ₂	CO ₂
0	2.916	63.34	34.87	1.78
4	2.666	63.53	34.77	1.69
10	2.356	63.83	34.59	1.57
15	2.150	64.04	34.51	1.44
20	1.983	64.34	34.30	1.36
25	1.845	64.76	33.93	1.30
30	1.729	65.18	33.60	1.21
35	1.621	65.19	33.62	1.17

Thus for a temperature rise of 10° C. (25° to 35° C.) while the total volume of dissolved air is decreased by 12.14 per cent., the percentage of this occupied by oxygen is reduced by 0.31 per cent., i.e. by 0.91 per cent. of its value at the lower temperature and that for nitrogen is increased by 0.43 per cent., i.e. by 0.66 per cent., whereas that for carbon dioxide is reduced by 0.13 per cent., i.e. by 10 per cent. This is a direct result of the higher temperature coefficient of solubility of carbon dioxide.

IV. THE SOLUBILITY OF DIFFERENT GAS MIXTURES IN WATER

(a) *The gases found in the internal atmospheres of fruits.*

It has been found (Wardlaw and Leonard, 1936, 1938) that in the internal cavity of hollow fruits such as the papaw, and in the air-spaces of the fleshy tissues of other tropical fruits, there exist gaseous atmospheres susceptible of analysis by simple methods, (i) differing considerably from that of the outside air, and (ii) showing a definite trend during the processes of development, maturation, and senescence of the fruit. These internal concentrations must stand in some definite relation to the concentration of the gases in the directly communicating intercellular spaces, and these in turn to the gases contained in the tissues themselves. It is unlikely that the relationship is the relatively simple one that exists between a gas and water or a solution of the same composition as the cell contents. Further, in studying respiration over a period of time, particularly in a detached organ, changes in the cell contents will occur and alter the solubility. Nevertheless, in attempting to analyse the basic relationship, the equilibrium between the nitrogen, oxygen, and carbon dioxide in the free gaseous condition and as dissolved gases in water, at different temperatures, suggests itself as an essential preliminary study.

The internal gas concentrations in developing and ripening fruits, ascertained in earlier studies, show that during development from half size to the onset of senescence the internal atmosphere is composed of nitrogen 79 per cent. and oxygen *plus* carbon dioxide 21 per cent., changes in oxygen and carbon dioxide concentrations being complementary. (Departures from this simple relationship occur in very young and again in over-ripe papaws, the sum of oxygen and carbon dioxide being less than 21 per cent.; again, in other fruits, values of O₂ *plus* CO₂ greater than 21 per cent. occur; these will be considered in later papers.) Table III has been constructed on this basis for a temperature of 25° C. and gives the volumes of oxygen and carbon dioxide dissolving in 100 c.c. of water in equilibrium with different gas mixtures, the total pressure being 760 mm. Nitrogen, which is present in constant gaseous concentration (79.04 per cent.), i.e. at constant partial pressure, maintains a constant value of 1.195 c.c.

The most striking feature of the relationship (Table III) is the preponderating influence on the total volume of dissolved gases of a slight increase in the percentage of carbon dioxide in the atmosphere. This is due to the relatively high solubility of carbon dioxide. An increase from 0.03 to 1 per cent. of carbon dioxide in the atmosphere causes an increase of 0.751 c.c. in the total volume of dissolved gas, i.e. of 40.70 per cent., whilst the colligative decrease in the volume of dissolved oxygen due to a reduction in concentration in the atmosphere from 20.93 to 20 per cent. is 0.027 c.c., i.e. 4.31 per cent.

Considering again, as was done in Table II for air, the percentage of the total volume of dissolved gas represented by the different constituent gases in these mixtures the data shown in Table IV are obtained.

TABLE III

The Volume (c.c.) of the Constituent Gases dissolving in 100 c.c. of Water (i.e. C_A) in Equilibrium with Different Gas Mixtures [$N_2 = 79.04\%$, ($O_2 + CO_2$) = 21%] at 25° C.

Oxygen		Carbon dioxide		$O_2 + CO_2$	$N_2 + O_2 + CO_2$
Gas conc.	C_A	Gas conc.	C_A	C_A	C_A
20.93 (air)	0.626	0.03 (air)	0.024	0.650	1.845
20	0.599	1	0.802	1.401	2.596
19	0.569	2	1.605	2.174	3.369
18	0.593	3	2.407	2.946	4.141
17	0.509	4	3.210	3.719	4.914
16	0.479	5	4.013	4.492	5.687
15	0.449	6	4.816	5.265	6.460
14	0.419	7	5.618	6.037	7.232
13	0.389	8	6.421	6.810	8.005
12	0.359	9	7.224	7.583	8.778
10	0.299	11	8.831	9.130	10.325
8	0.239	13	10.43	10.669	11.864
6	0.180	15	12.04	12.220	13.415
4	0.120	17	13.65	13.770	14.965
2	0.060	19	15.25	15.310	16.505
1	0.030	20	16.05	16.080	17.275
$N_2 = 79.04\%$		1.195			

TABLE IV

Volumes of Constituent Gases expressed as Percentages of the Total Volume of Gas dissolved in Water in Equilibrium with Different Gas Mixtures [$N_2 = 79.04\%$, ($O_2 + CO_2$) = 21% at 25° C.]

Gas conc. (%)	O_2	CO_2	Percentage of total volume dissolved		
			N_2	O_2	CO_2
20.93(air)	0.03		64.76	33.93	1.30
20	1		46.03	23.08	30.89
19	2		35.46	16.89	47.64
18	3		28.85	13.02	58.12
17	4		24.31	10.35	65.31
16	5		21.01	8.42	70.57
15	6		18.49	6.95	74.54
14	7		16.52	5.79	77.69
13	8		14.92	4.86	80.21
12	9		13.61	4.09	82.28
10	11		11.56	2.90	85.48
8	13		10.07	2.02	87.94
6	15		8.90	1.34	89.73
4	17		7.98	0.80	91.18
2	19		7.24	0.36	92.34
1	20		6.92	0.17	92.90

This table brings out the great effect at the higher values of a small reduction in the gaseous concentration of oxygen on the percentage of the total volume of dissolved gases occupied by it. Thus a reduction of

oxygen concentration from 20.93 to 19 per cent., i.e. less than 2 per cent., with concomitant increase in the carbon-dioxide concentration, reduces the dissolved oxygen expressed as percentage of the total dissolved gas to about half (from 33.9 to 16.9 per cent.), although the reduction in volume is only 0.057 (0.626—0.569) c.c. or 9.1 per cent. of the higher value. At intermediate and lower values of gaseous oxygen concentration the effect is much

TABLE V

Values of Internal Concentrations of O₂ and CO₂ in the Cavity of the Papaw during Development and Ripening, and the Volumes of these Gases dissolved in 100 c.c. of Water in Equilibrium with such Concentrations at 25° C.

Stage	Internal conc. %		Vol. (c.c.) of gas in 100 c.c. of water				
	O ₂	CO ₂ (O ₂ +CO ₂)	O ₂	CO ₂	(O ₂ +CO ₂)		
Development	1	13.5	5.2	18.7	0.404	4.174	4.578
	2	16.4	3.4	19.8	0.491	2.728	3.219
	3	17.5	2.5	20.0	0.524	2.006	2.530
	4	18.1	2.2	20.3	0.542	1.765	2.307
	5	18.5	2.0	20.5	0.554	1.605	2.159
	10	18.8	1.9	20.7	0.563	1.525	2.088
	11	18.8	2.2	21.0	0.563	1.765	2.328
	11.5	17.4	3.6	21.0	0.521	2.889	3.410
	12	15.4	5.6	21.0	0.461	4.495	4.956
	12.5	12.5	8.4	20.9	0.374	6.742	7.116
Ripening and Senescence	13	9.0	11.2	20.2	0.335	8.991	9.326
	13.5	2.6	14.2	16.8	0.078	11.396	11.474
	14	0.2	16.4	16.6	0.006	13.167	13.173
	14.5	0.0	16.8	16.8	0.0	13.489	13.489
	15	0.0	18.8	18.8	0.0	15.090	15.090
Air (N ₂ = 79.04%)	20.93	0.03	20.96	0.626	0.024	0.650	

less marked, a reduction from 12 to 10 per cent. causes a reduction of only 1.19 per cent. (4.09—2.90 per cent.) of the total dissolved gas although the reduction in volume is the same (0.359—0.299 = 0.060 c.c.), and represents the same percentage reduction, 16.7 per cent., of the value at the higher gaseous concentration.

As an example of the application of these tables to biological data the values of the internal concentrations (i.e. those in the cavity) of oxygen and carbon dioxide during the development, maturation, and senescence of the papaw at 77° F. (25° C.) as determined in earlier experiments (Wardlaw and Leonard, 1936, 1938) are reproduced in columns 2, 3, and 4 of Table V and as a graph in Fig. 1. The stages of development, maturation, and senescence are on an arbitrary basis. For an estimate of development (stages 1 to 10) weight is taken, while for maturation and senescence (stages 11 to 15) time is taken. The points at which the peak of the climacteric in the respiration rate is attained are indicated, as are also the first appearance of anthracnose spotting and the onset of severe fungal wastage.

If a simple gas/water system alone was involved, the quantities of dissolved

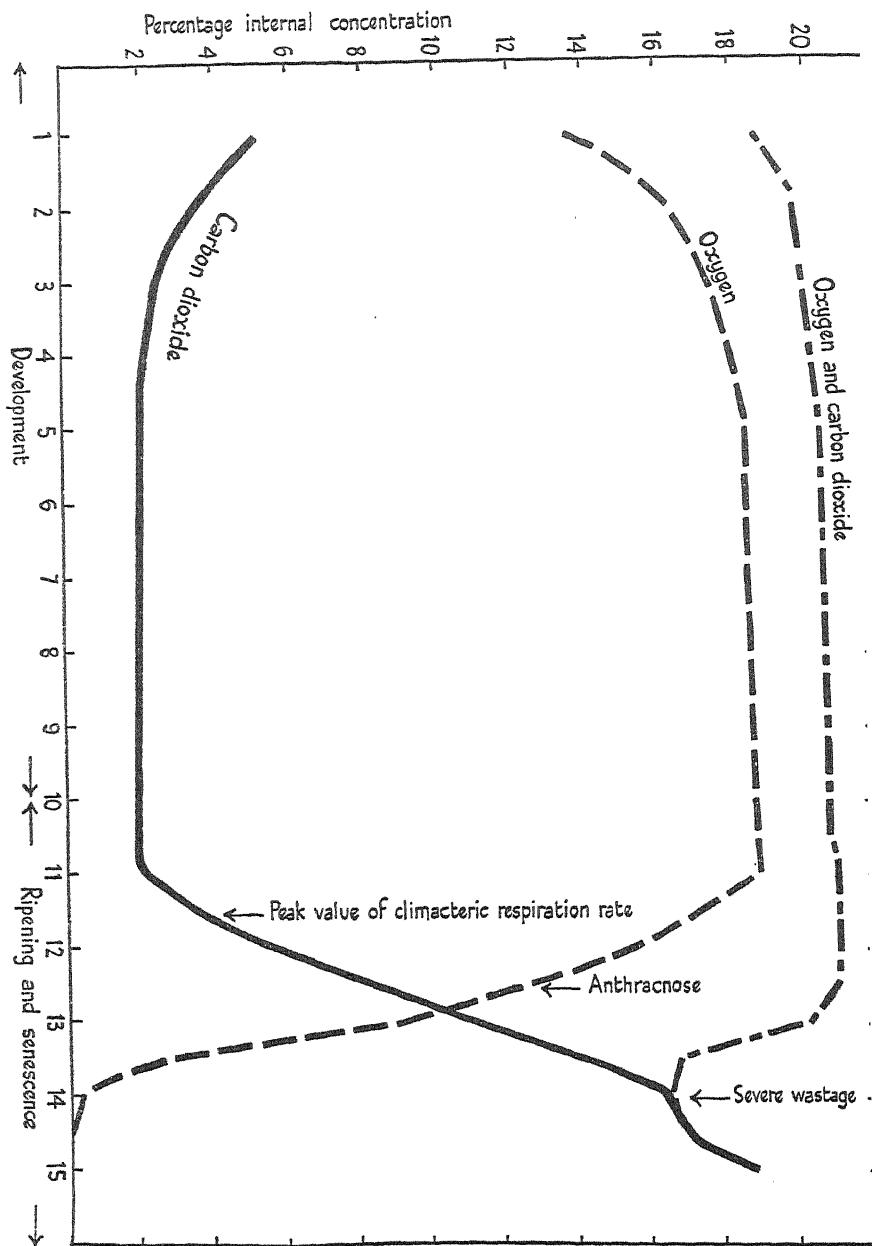


FIG. 1. Internal gas concentrations during the development, maturation, and senescence of the papaw at 25°C .

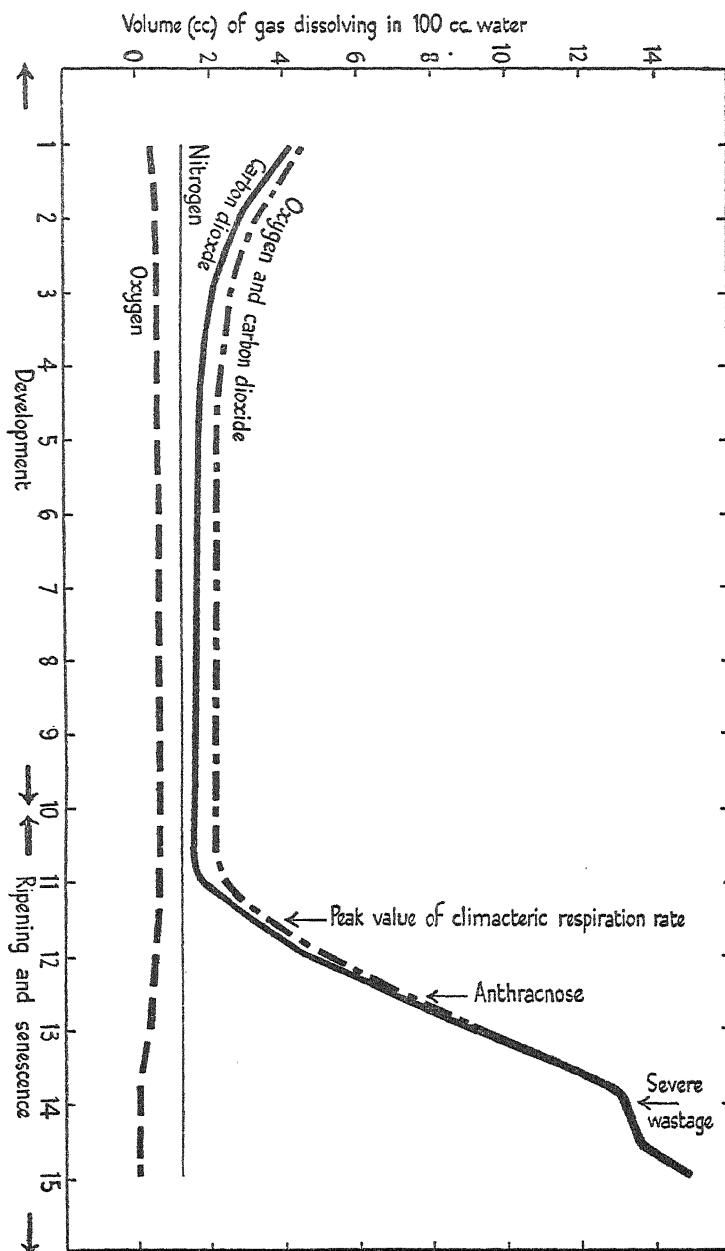


FIG. 2. Volumes of gases dissolving in 100 c.c. water in equilibrium with internal gas concentrations found in the cavity of the papaw at 25° C.

oxygen and carbon dioxide at the different stages would be those shown in the last three columns of Table V. These values are reproduced in Fig. 2. The small volume of dissolved oxygen and the large volume of dissolved carbon dioxide relative to their respective gaseous concentrations throughout the development of the papaw will be seen and also the large increase in the volume of dissolved carbon dioxide during the climacteric rise and subsequently in the senescent fruit. These features are emphasized in Table VI which gives the total volume of dissolved gas under the same conditions, and the percentages of this total volume occupied by each of the constituent gases. The latter values are shown graphically in Fig. 3.

TABLE VI

Total Volume of Dissolved Gas in 100 c.c. of Water at 25° C. in Equilibrium with Gas Mixtures as in Developing and Maturing Papaw, and Volumes of the Constituent Gases expressed as Percentages of this Total Volume

Stage	Total volume of dissolved gas	Percentage of total volume			
		N ₂	O ₂	CO ₂	(O ₂ + CO)
Development	1 5.773	20.69	7.00	72.30	79.30
	2 4.414	27.07	11.13	61.81	72.94
	3 3.725	32.07	14.07	53.86	67.93
	4 3.502	34.12	15.47	50.40	65.87
	5 3.354	35.63	16.52	47.86	64.38
	10 3.283	36.39	17.15	46.44	63.59
	11 3.523	33.91	15.98	50.09	66.09
	11.5 4.605	25.94	11.31	62.73	74.04
	12 6.151	19.42	7.49	73.07	80.56
	12.5 8.311	14.37	4.50	81.12	85.62
Ripening and Senescence	13 10.521	11.36	3.18	85.47	88.65
	13.5 12.669	9.43	0.62	89.97	90.59
	14 14.368	8.31	0.04	91.64	91.68
	14.5 14.684	8.13	0.0	91.87	91.87
	15 16.285	7.34	0.0	92.66	92.66
Air	1.845	64.77	33.93	1.30	35.23

It will be seen that whereas the sum of the percentage internal gaseous concentrations of oxygen and carbon dioxide amounts to 20.5–21 per cent. in papaws which have reached half their final size (stage 5) and remains at this value during the earlier stages of maturation (to stage 12.5), the sum of the volumes of these two gases expressed as a percentage of the total volume of dissolved gas ranges from 64 to 85 per cent., the lowest value being attained immediately prior to the climacteric rise in internal gaseous carbon-dioxide concentration when this gas is at its lowest and oxygen at its highest value.

A further example of the application of these tables is found in the 'transition effect' which occurs in the rate of liberation of carbon dioxide from plant organs when transferred from an atmosphere of high to one of low carbon-dioxide concentration. This has been the subject of comment by Spoehr

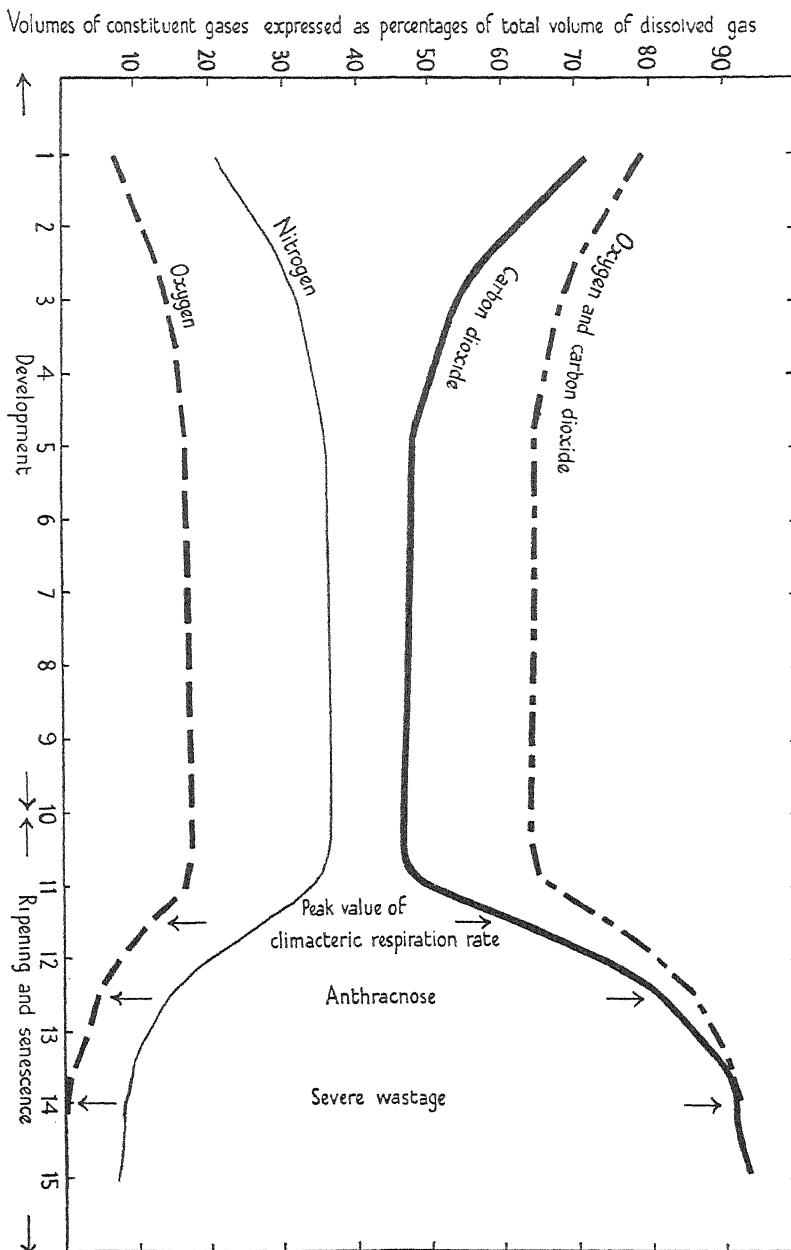


FIG. 3. Volumes of the constituent gases expressed as percentages of the total volume of gas dissolving in water in equilibrium with internal gas concentrations found in the cavity of the papaw at 25° C.

and McGee (1924), Blackman and Parija (1928) and Wardlaw (1936). The carbon dioxide retained in the tissues as a result of the high external atmospheric concentration is rapidly liberated until a fresh equilibrium is established, producing an apparent rise followed by a fall in the rate of respiration which may in part be ascribed to the change in gradient of carbon dioxide. In a

TABLE VII

Volumes (c.c.) of Gases dissolving in 100 c.c. of Water in Equilibrium with Different Gas Mixtures [N₂ = 79·04%, O₂+CO₂ = 21%] at 15° and 35° C.

Conc. of gas (%)	O ₂		Conc. of gas (%)	CO ₂		N ₂ +O ₂ +CO ₂	
	15° C.	35° C.		15° C.	35° C.	15° C.	35° C.
20·93 (air)	0·742	0·545	0·03 (air)	0·031	0·019	2·150	1·621
20	0·710	0·520	1	1·056	0·631	3·143	2·208
19	0·674	0·494	2	2·113	1·262	4·164	2·813
18	0·638	0·468	3	3·171	1·893	5·186	3·418
17	0·603	0·442	4	4·227	2·525	6·207	4·024
16	0·568	0·416	5	5·284	3·156	7·229	4·629
15	0·532	0·390	6	6·342	3·787	8·250	5·234
14	0·497	0·364	7	7·398	4·420	9·272	5·841
13	0·461	0·338	8	8·455	5·049	10·293	6·444
12	0·426	0·312	9	9·513	5·680	11·316	7·049
10	0·355	0·260	11	11·62	6·942	13·352	8·259
8	0·284	0·208	13	13·74	8·206	15·401	9·471
6	0·213	0·156	15	15·85	9·464	17·440	10·677
4	0·142	0·104	17	17·96	10·73	19·479	11·891
2	0·071	0·052	19	20·08	11·99	21·528	13·099
1	0·035	0·026	20	21·13	12·62	22·542	13·703
N ₂ (79·04%)	1·377	1·057					

physical system this 'transition effect' would be wholly due to this change; in a biological system, however, there may be a rise in the rate of carbon-dioxide production accompanied by an increase in the internal concentration of this gas, so that a new level of respiration is reached in the altered environment, i.e. in an external atmosphere of lower carbon-dioxide and higher oxygen concentration.

A further difference found in biological systems is that a 'transition effect' is generally observed when a plant organ is transferred from air to nitrogen. Here no appreciable change in gradient of carbon-dioxide concentration is involved, the plant organ passing from an atmosphere of (79·04 per cent. N₂+20·93 per cent. O₂+0·03 per cent. CO₂) to one of (100 per cent. N₂). Other than purely physical considerations are presumably involved in this case.

(b) The influence of temperature.

In a biological system the influence of temperature is seen in both chemical and physical relationships and in the interactions between them. Considering, for the moment, only the effect of temperature on the solubility of gases we have in Table VII the volumes of oxygen and carbon dioxide dissolving in

100 c.c. of water at 15° C. and 35° C. in equilibrium with the same gaseous concentrations of these gases and nitrogen as were employed in Table III. These values give curves similar to those given by the data of Table III and, if the volumes of the constituent gases expressed as percentages of the total volume of dissolved gas are calculated and plotted against gaseous concentration, useful curves are obtained.

TABLE VIII

Volumes (c.c.) of Gases liberated from 100 c.c. of Water resulting from a Rise in Temperature of 10° C., the Water being in Equilibrium with Different Gas Mixtures [N₂ = 79·04%, O₂+CO₂ = 21%]

Conc. of gas (%)	15° to 25° C.						25° to 35° C.		
	O ₂	CO ₂	O ₂	CO ₂	N ₂ +O ₂ +CO ₂	O ₂	CO ₂	N ₂ +O ₂ +CO ₂	
20·93 (air)	0·03	0·116	0·007	0·305	0·081	0·005	0·224		
20	1	0·111	0·254	0·547	0·079	0·171	0·388		
19	2	0·105	0·508	0·795	0·075	0·343	0·556		
18	3	0·099	0·764	1·045	0·071	0·514	0·723		
17	4	0·094	1·017	1·293	0·067	0·685	0·890		
16	5	0·089	1·271	1·542	0·063	0·857	1·058		
15	6	0·083	1·526	1·790	0·059	1·029	1·226		
14	7	0·078	1·780	2·040	0·055	1·198	1·391		
13	8	0·072	2·034	2·288	0·051	1·372	1·561		
12	9	0·067	2·289	2·538	0·047	1·544	1·729		
10	11	0·056	2·789	3·027	0·039	1·889	2·066		
8	13	0·045	3·31	3·537	0·031	2·224	2·393		
6	15	0·033	3·81	4·025	0·024	2·576	2·738		
4	17	0·022	4·31	4·514	0·016	2·92	3·074		
2	19	0·011	4·83	5·023	0·008	3·26	3·406		
1	20	0·005	5·08	5·267	0·004	3·43	3·572		
N ₂ (79·04%)		0·182			0·138				

Owing to the decreased solubility of all the three gases at higher temperatures, various quantities of gas will be released from solution on passing from a lower to a higher temperature. The volume of each gas so liberated depends upon (i) the amount dissolved, which in turn is determined by the gas mixture, upon (ii) the temperature rise, and upon (iii) the temperature coefficient of solubility of the individual gas. Table VIII has been constructed from Tables III and VII to show the volumes of the three gases released for a rise in temperature of 10° C. at two initial temperatures (15° to 25° and 25° to 35° C.) and at the gas concentrations previously considered. The volume of each gas liberated is greater at the lower temperature change (15°–25° C.). The volume of (O₂+CO₂) and of total gas liberated increases with increasing volumes of carbon dioxide in solution.

If the volume of each gas liberated due to the rise in temperature is expressed as a percentage of the volume of that gas dissolved at the lower temperature, it is found (i) that there is a slight increase in the percentage

reduction in volume of oxygen and a decrease in the percentage reduction in volume of carbon dioxide at the highest levels of gaseous oxygen concentration and vice versa at the lowest levels, but that over the greater part of the range of gaseous concentrations ($O_2 = 20$ per cent., $CO_2 = 1$ per cent. to $O_2 = 2$ per cent., $CO_2 = 19$ per cent.) there is a constant percentage reduction in volume of each gas, (ii) that owing to the different temperature coefficients of solubility the percentage reduction in dissolved nitrogen is less than that in dissolved oxygen which, in turn, is less than that in carbon dioxide, (iii) that there is an increasing percentage reduction in dissolved ($O_2 + CO_2$) with increasing proportions of carbon dioxide which is reflected in the percentage reduction in volume of the total gas liberated.

Turning to a consideration of the effect of a rise in temperature on biological systems, such a rise in the rate of liberation of carbon dioxide from a plant organ has been noted by all workers on respiration. Where observations are made at frequent intervals it is generally found to be followed by a fall to a fresh level, i.e. there is a 'transition effect' which appears as a peak between the two levels of respiration rate. This 'transition effect' has been discussed by De Long, Beaumont and Willaman (1930), Willaman and Brown (1930) and Miller and Burr (1935) who ascribe it to the release of dissolved carbon dioxide from the tissues. The actual volume of this gas liberated during this 'transition period' cannot be referred solely and quantitatively to the decreased solubility at the higher temperature as would be the case in a purely physical system. The rate of production of carbon dioxide at what have been described as 'respiratory centres' also increases with temperature, as also does the concentration of carbon dioxide in the intercellular spaces of the plant organ (Wardlaw and Leonard, 1936). Kidd (1914), however, suggests that the partial pressure of carbon dioxide (in seed embryo tissue) is the same at all temperatures. The appreciable increase in pulp temperature during the climacteric (Wardlaw and Leonard, 1939) will itself modify the curve of carbon dioxide liberation during what may be regarded as a major transition effect. If the volume of carbon dioxide dissolving in water at the higher temperature and in equilibrium with the increased internal concentration is calculated (using values for the internal concentration ascertained in experiments, (Wardlaw and Leonard 1936)), it is found to be greater than that at the lower temperature. The 'transition effect' in the rate of liberation of carbon dioxide is therefore less marked when the rise in temperature of the tissues is slow, the physical effect of decreased solubility being offset by the increased internal concentration.

(c) *The influence of pressure.*

Some consideration of the effect of barometric pressure on the amount of dissolved gas is necessary. The diurnal range of barometric pressure in Trinidad is from 759.5 to 764.5 mm. Hg about an average value of 762 mm. By calculation it is found that over this range of pressure the volumes of gases

dissolving in water are not affected by more than 1·4 per cent. of the volume dissolved at the mean pressure.

In the third contribution in this series (Wardlaw and Leonard, 1938) differences in pressure between the atmosphere and the gases in the inter-cellular spaces or central cavities of fruits have been the subject of preliminary investigations. For example, it has been observed that the maximum range of pressure recorded by manometers attached to the internal cavity of papaws is from 16 cm. of liquid paraffin below to 1 cm. above the atmospheric pressure, i.e. equivalent to a range of 761·90 to 762·07 mm. of mercury about the mean barometric pressure. Although this is slightly less than the diurnal barometric range given above, and is independent of it, nevertheless it is seen to be important.

While considerable attention has been paid in physiology to osmotic pressure, its effect on gas-solution phenomena appears to be still unexplored. It is evident that this aspect of gas solution requires careful investigation in relation to biological phenomena.

(d) Interaction of temperature and pressure.

A further consideration is the interaction of temperature and pressure. Thus in Trinidad it is found that the diurnal cycle of a rise and then a fall in temperature is accompanied by a fall and rise in barometric pressure. There is therefore a certain amount of augmentation of the amounts of dissolved gases, increasing pressure, and decreasing temperature during the night producing an increase and decreasing pressure and increasing temperature during the day producing a decrease. The barometric range gives only a small change, 0·5 cm. in 76·2, or approximately 1 in 150, corresponding to a temperature change of about 2°C. (298° Abs./150) about the mean 25°C.¹ By contrast, the diurnal range of shade temperature is of the order of 10°C., i.e. 10/298, or 1 in 30, about five times that of pressure. The physical calculations in this paper have been based on a constant pressure of 760 mm.

V. THE SOLUBILITY OF OTHER GASES

Whilst the three gases so far considered are those most frequently present and in greatest quantity in all biological systems, the presence, in even minute quantity, of certain gases has been found to exercise considerable effects on the respiration of plants. These have been studied particularly in the storage of fruits, where the addition of a trace of such gases as ethylene may produce marked effects on the rate of liberation of carbon dioxide simultaneously with application or shortly after.

It is to be noted that ethylene is moderately soluble in water. The solubility

¹ In *biological* systems, however, a temperature difference of 2°C. is very important inasmuch as it affects metabolism as well as gas solubility.

of ethylene at 25° C. is given in Table IX with those for the other three gases for comparison.

TABLE IX

Volume (c.c.) of Gas dissolving in 100 c.c. of Water at 760 mm. and 25° C.

CO ₂	75·9
C ₂ H ₄	10·8
O ₂	2·8
N ₂	1·4

It is noteworthy that other gases such as ammonia and hydrochloric acid gas which produce marked physiological responses when present in very small quantities are very soluble in water.

VI. THE SOLUBILITY OF GASES IN LIQUIDS OTHER THAN WATER

While water is the predominant solvent in biological systems other liquids may also be present in small quantities in plant tissues and may play some part as solvents for gases. Table X gives the solubility in alcohol of the three gases, nitrogen, oxygen, and carbon dioxide, and also of ethylene. The relatively large volumes of all these gases in solution is noticeable, particularly those of carbon dioxide and ethylene.

TABLE X

Volume (c.c.) of Gas dissolving in 100 c.c. Ethyl Alcohol at 760 mm. and 25° C.

CO ₂	275·6
C ₂ H ₄	257·8
O ₂	21·8
N ₂	12·0

It must be noted, however, that published data on the solubility of gases in mixtures of alcohol and water indicate that for oxygen and carbon dioxide, at least, there is a minimal solubility with increasing amounts of alcohol in water.

VII. THE INFLUENCE OF SOLUTES ON THE SOLUBILITY OF GASES

In general the effect of the presence of solutes is to decrease the solubility of gases. This phenomenon has been the cause of considerable research in attempts to determine the general law underlying it. Figs. 4 and 5 give values for the solubility of nitrogen, oxygen, and carbon dioxide in solutions of glucose, sucrose, starch, and dextrin. (Published data for the solubility of carbon dioxide in gelatin show considerable variability, but there appears to be a slight increase with increasing concentration.) These observations have particular interest and application in the ripening of fruits when well-marked changes are taking place in gas and sugar concentrations. Again, it may be emphasized that considerably different equilibria between gases and solvents may exist in physical and biological systems.

VIII. SUMMARY

1. The importance of the solubility of gases in relation to respiration is considered.
2. The volumes of the gases chiefly involved in respiration, and dissolving

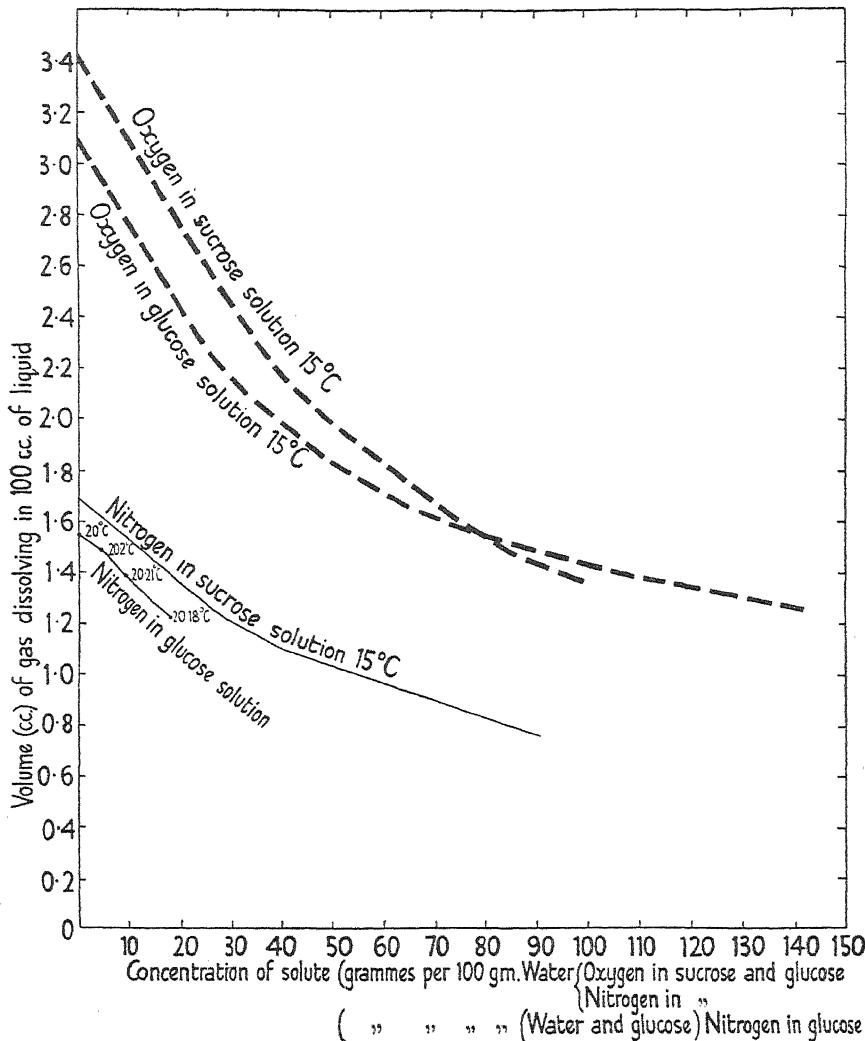


FIG. 4. Volumes of nitrogen and oxygen dissolving in solutions of glucose and sucrose.

in water at the partial pressures (percentage concentrations), total pressures, and temperatures most frequently encountered under biological conditions, are given in the form of tables and graphs.

3. Tables and graphs are provided showing the percentage of the total volume of gas in solution occupied by each constituent gas and of the volumes of these gases liberated from such solution with rise in temperature.

4. Data are also given of the solubility of other gases in water, of nitrogen,

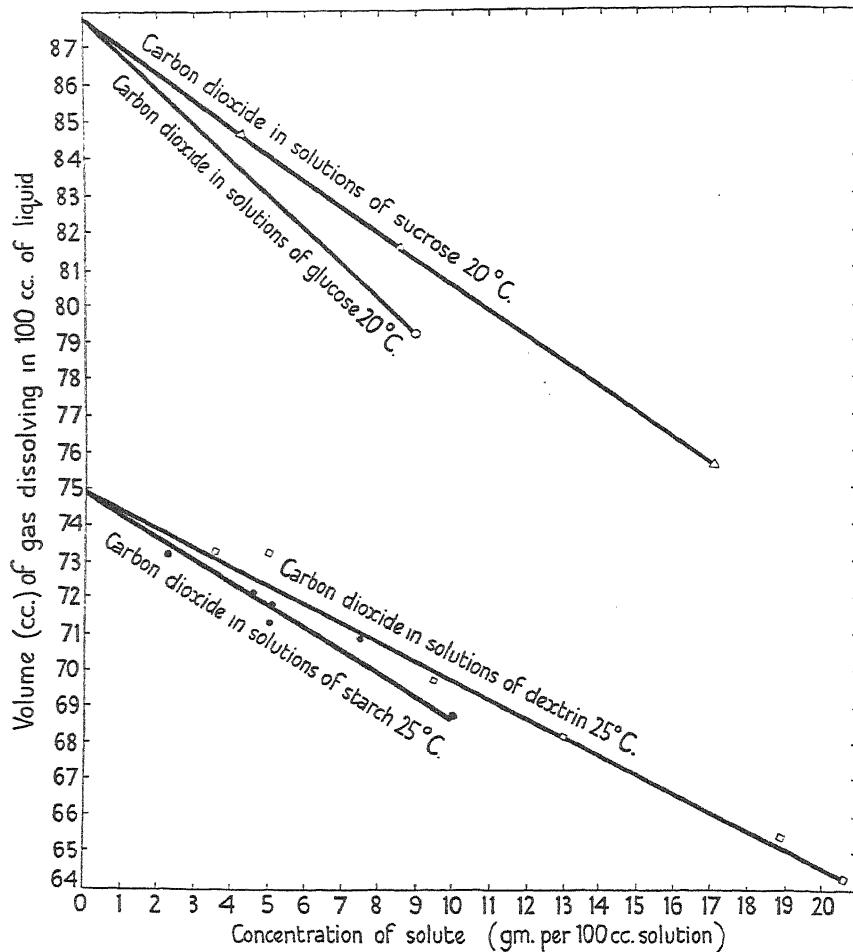


FIG. 5. Volumes of carbon dioxide dissolving in solutions of glucose, sucrose, starch, and dextrin.

oxygen, and carbon dioxide in solvents other than water, and of the effect of solutes on the solubility of these gases.

5. An example is given of the application of these tables based on values of gas concentrations observed in respiration studies of the papaw.

6. It is emphasized that the relatively simple relationships between a gas or mixture of gases and water are unlikely to obtain in biological systems, but

a knowledge of these relationships is essential to the analysis of such systems. Instances are given of differences between the two systems.

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Studies in Tropical Fruits

VII. Notes on Banana Fruits in Relation to Studies in Metabolism

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With six Figures in the Text

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I. INTRODUCTION

THE banana not only ranks as one of the major tropical crops but has also a particular interest for botanists in the rapid and considerable changes which take place in the bunch during its development on the plant and ripening in storage. Studies in the physiology and biochemistry of the banana have been undertaken as a means of elucidating the trend of metabolic changes during ripening. Almost without exception, previous investigations have, of necessity, been conducted on fruit which has already been subjected to refrigerated storage during overseas transport: as a result the data which have been obtained, as well as being unavoidably incomplete, are based on fruits whose previous history is insufficiently known and in which more or less extensive but undetermined metabolic changes have already taken place. The practical and scientific value of studies carried out in the tropics, and based on freshly harvested fruit whose cultivation history is known, has been appreciated for some time.¹ This work is now in progress, and will be

¹ The following quotations from the Annual Reports of the Food Investigation Board for 1933 and 1935 illustrate this point: 'There is much fundamental work required for the needs of our own people that cannot be carried out here because the experimental material is unobtainable, and it is therefore our task to promote this where it can best be done; an

published in due course in this series. The purpose of the present notes is to give some account of (i) the ontogenetic development of the banana bunch and fruit to maximum size, and (ii) the selection of materials for use in physiological and biochemical studies.

II. ONTOGENETIC DEVELOPMENT AND MORPHOLOGY OF THE BUNCH

Seedless varieties of bananas, of which the Gros Michel is the most important commercially, have been propagated by vegetative means from prehistoric times. They have a very condensed but profusely branched underground stem or rhizome—which is a storage organ. The 'eyes' or buds on developing give rise to suckers. With further growth these develop into plants consisting of an elongated trunk or pseudo-stem, composed of closely packed, overlapping leaf bases, and a crown of foliage. As in many other Monocotyledons, the true apex of each stem or trunk is, therefore, during the greater part of the growth of the individual sucker or tiller, at or below soil level and in close continuity with the storage rhizome. In due course, as leaf formation approaches completion, the inflorescence develops from the apical meristem which is used up in the process. According to the nutritional status of the rhizome at the time of formation of the flowering shoot, an embryonic bunch of few or many 'hands' may be initiated. The flowers are in clusters or hands, each consisting of two rows, the hands being arranged spirally on the stalk.

Three types of flower can be distinguished. Those at the base of the stalk with long ovaries become the individual fruits or 'fingers' of the bunch. Higher up are hermaphrodite flowers with noticeably shorter ovaries, which remain short and undeveloped. At the apex of the shoot are clusters of male flowers. Each flower cluster is closely ensheathed in a bract. In the initial stage, therefore, the young inflorescence consists of a compact bud, projecting from the rhizome into the base of the trunk or pseudo-stem at about soil level.

According to Fawcett (1913), 'the actual length of time which is necessary from the first planting of the bulb (or piece of rhizome) until the period arrives when the plant commences flower formation will vary according as

obvious example is the physiology of tropical and semi-tropical fruits, and we venture to take this opportunity of remarking that we should welcome more attention, in this respect, to those two important fruits, the orange and the banana.' (1933.)

'We would call attention to an inevitable omission from the programme of research needed by the interests of the consumer in the United Kingdom. The apple, the orange and the banana together account for some three-quarters of his consumption of raw fruit, the consumption of apples and oranges being roughly equal, and that of bananas about one-half as much. Fundamental research on the orange and banana cannot be carried out in this country; it must be done where these fruits are grown. Research on both these fruits is being done in various parts of the Empire; on the orange, notably in Australia and South Africa, and on the banana, in Australia and the West Indies. But in the case of the banana much more is required, in the interest of the consumer in the United Kingdom, than is yet being undertaken. The West Indies supply practically the whole of our imports of bananas from the Empire, the quantity being roughly one-half of the total supply.' (1935.)

the conditions for growth have been favourable or otherwise. But probably the time may be safely put down as somewhere between seven and nine months.' With further growth, the inflorescence stalk (which is in fact the true vertical shoot or stem of the individual banana plant) begins to elongate, so that the inflorescence bud is thrust upwards under considerable pressure through the narrow central channel of the pseudo-stem. When the bud eventually emerges at the crown of the plant it is said to be 'shot'. The time that elapses between inflorescence formation at soil level and emergence at the foliage crown has not been precisely determined. According to Fawcett (1913) it 'may possibly be as short as one month or as long as two months', water-supply being considered an important factor.

With further elongation of the inflorescence shoot, or as it may now be described, the main-stalk of the bunch, the inflorescence bud begins to hang over and the bracts to open and fall, thereby disclosing in succession the female flower clusters or hands. During this period the rapid growth of the inflorescence stalk continues so that the 'hands' become separated by several centimetres of stalk. Beyond the hermaphrodite flowers, the bracts and male flowers open and fall in acropetal succession, so that eventually a considerable length of stalk separates the male bud from the bunch proper. If growth continues to take place rapidly at this stage the individual banana fruits or fingers begin to fill out and the collective weight bends the main-stalk over so that the bunch hangs in a vertical position thereby permitting of symmetrical development. Any curtailment of growth at this stage may result in the bunch remaining in a semi-horizontal position for some time, in consequence of which the negative geotropism of fingers makes for the development of an asymmetrical bunch.

The development of the bunch from the time it is 'shot' till it attains to ' $\frac{3}{4}$ -full' or 'heavy $\frac{3}{4}$ -full' size may occupy approximately three to four months.¹ Immediately after the bunch has been shot and the bracts opened to disclose the hands, very marked differences in the distribution and number of hands, the number of fruits per hand, and the length of individual fruits may be observed. These are related to growth-conditions and other factors considered in the next Section.

The number of fingers per hand is variable, the number increasing as the number of hands increases. According to Hartman (1929), cited by Kervegant (1935), there is a minimum of thirteen fingers per hand in a six-hand bunch of Gros Michel; for each additional hand there is an increase of one finger per hand up to ten hands, and of a half finger per hand thereafter.

The female flower which, as is well known, undergoes further development without requiring the stimulus either of pollination or fertilization, consists of an elongated, inferior, trilocular ovary of three fused carpels, a five-lobed tubular perianth of three sepals and two petals, all united, and one small free

¹ The terms ' $\frac{3}{4}$ -full', &c., are discussed in Section III.

petal, and a defective androecium. The perianth, androecium, and style soon become withered and discoloured, but may remain attached for some time more or less completely isolated from the ovary by a brownish corky layer. In commercial varieties the ovules normally remain unfertilized and at an early stage become brown-coloured and abortive.

Anatomical and histological studies referring to the details of fruit structure have been summarized by Kervegant (1935). Further work is being undertaken at this Station on this subject.

III. CAUSES OF VARIABILITY

The banana of commerce is cut whilst quite green, whether intended for local consumption or long-distance transport, descriptive terms such as '½-full', 'heavy ¾-full', and 'full' being used to indicate the maturity or grade of bunches at harvesting. In practice the assessment of grade to ensure that the storage life required in particular instances will be obtained is purely arbitrary. As the conditions under which the Gros Michel banana is cultivated show wide variations both in respect of soil, rainfall, and agricultural practice, harvesting maturity is subject to variation and is based on local experience. In general, bananas grown under conditions of continuous rainfall tend to yield large bunches and fruits, whereas under drier conditions bunches and individual fruits tend to be smaller. It follows that actual size alone does not provide an accurate guide to maturity except where bunches are being produced under closely comparable conditions. Again, under different cultural conditions, bunches may require a longer or shorter time to reach a given size. In such instances it may be of great importance to distinguish between chronological and physiological age; in this connexion reference may be made to the work of Gustafson (1929) on tomatoes, in which he indicates that the two terms are not necessarily synonymous.

In banana bunches selected for physiological study and judged by size and appearance as being closely comparable, considerable differences may, in fact, be present. These are referable to one or more of the following factors: (a) edaphic conditions, permitting of rapid, intermediate, slow or intermittent growth; (b) age, condition and nutritional status of the rhizome, the number of bunches developing concurrently from the rhizome being important; (c) closeness of planting (e.g. partial shade slows down the rate of development of the bunch); (d) harmful factors such as excessive wind action, fungal diseases, &c., which may reduce the effective leaf area.

Hartman (1929, 1930) has shown for Honduras fruit that the average weight of nine-hand bunches cut for export shows considerable variation from month to month and from year to year.

IV. DEVELOPMENTAL DATA

Samples were taken during the period June to August 1937 from the Montserrat district of Trinidad for a biochemical study of the banana during

development. A considerable amount of data on the weights of fingers and that of their constituent pulp and skin¹ was thus accumulated. From this a brief study is presented of the growth of the finger and of the pulp/skin weight ratio during development.

The records are divided into two series: (*a*) those based on bunches of known age (i.e. bunches which had been tagged at the time of 'shooting'), a separate bunch being used for each sample of fingers; (*b*) those based on samples obtained from plants which were judged to carry ' $\frac{3}{4}$ -full' (English grade) fruit on June 18 and were therefore, from general experience, given the arbitrary age of 80 days from the day on which they were shot; the age of bunches in this series is given in inverted commas in Table I. Three plants, A, B, and C, were selected and fingers from their bunches removed at frequent intervals until the fruit rotted or, in the case of C, the plant fell down. The fruit was protected from birds, bats, &c., by enclosing the bunches in bags.

In the case of series (*a*) the bunches were received at the laboratory within two or three hours of cutting and immediately sampled; in series (*b*) the fruit was sampled by cutting fingers from the bunch, and transporting them in sealed boxes to the laboratory where they were divided into skin and pulp and weighed within two hours of cutting.

The data for both series are set out in Table I and from the values given in columns 4 and 5 the curves in Fig. 1 have been constructed to show the weight of the whole finger and of the pulp during the development of the fruit. It will be observed that the weights both of the whole finger and of the pulp increased for the entire period over which the bunch remained attached to the plant and, in fact, in this particular instance, the rate of increase was accelerated after attainment of the level of maturity at which fruit is usually cut for the English market (' $\frac{3}{4}$ -full'). The 'heavy $\frac{3}{4}$ -full' grade, cut in Trinidad for the Canadian market, corresponds approximately to the stage of development at '90' days in Fig. 1, i.e. fruit of 150 to 160 gm. fresh weight per finger. After '100' days the skins of the fruit started to split due to the relatively greater rate of expansion of the pulp and also to the fact that metabolic changes akin to ripening had begun (biochemical data to be published later). The maximum size-limit for fruit cut with a view to storage was therefore given, for the fruit from this estate and this season, by the '100' day level of maturity, i.e. approximately 50 per cent. greater fresh weight of whole fingers and 60 per cent. greater pulp weight than the grade for the English market. Fruit cut beyond this stage would inevitably ripen before any appreciable storage period had elapsed.

Fig. 2 sets out the data of column 7 of Table I for the pulp/skin ratio

¹ The separation of skin from flesh at the different stages of development of green bananas, whilst not taking place along quite such a well defined layer as in the ripe fruit, is nevertheless fairly readily and uniformly accomplished. In every case the sample fingers were taken from the centre of the third or fourth hand of each bunch.

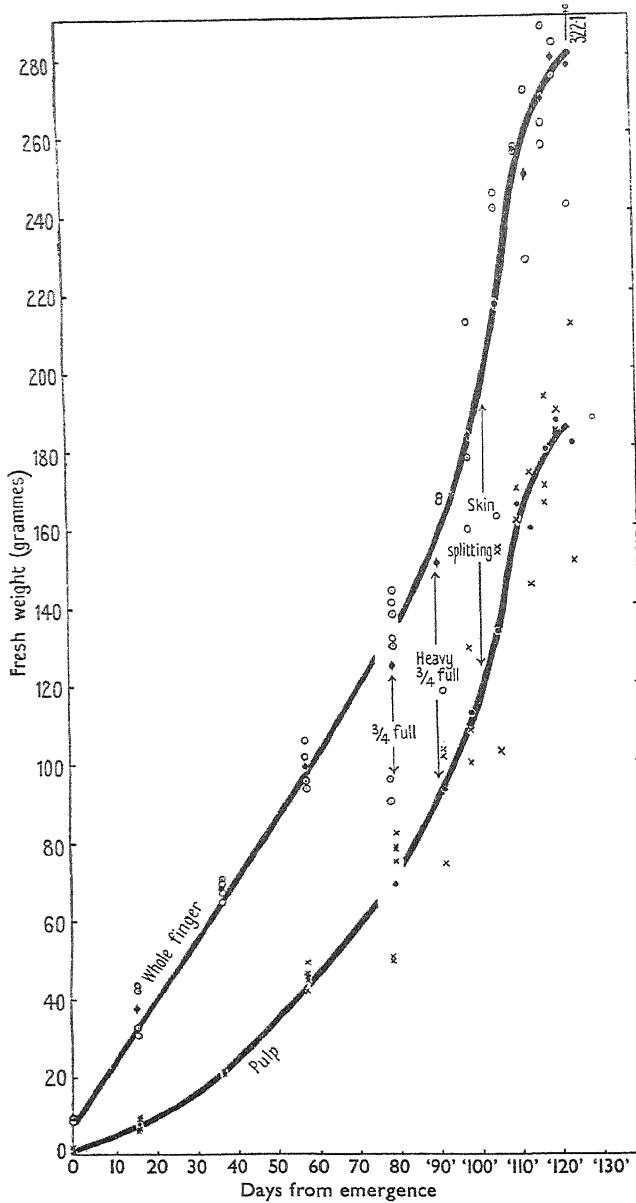


FIG. 1. The growth on the plant of the whole finger and of the pulp of the banana. Values for the fresh weights of whole fingers are shown by circles and for pulps by crosses. Approximations to smooth curves were obtained by plotting the means of the values obtained for samples of the same age (whole-finger-weight means indicated by dots with vertical lines drawn through them and pulp-weight means by dots); through these points smooth curves were drawn by eye.

The break in each curve indicates the end of the scale of actual ages of fingers in days from emergence of the bunch, and the beginning of the scale based on the arbitrary age of '80' days assigned to the bunches selected as having standard ' $\frac{3}{4}$ -full' English grade fruit.

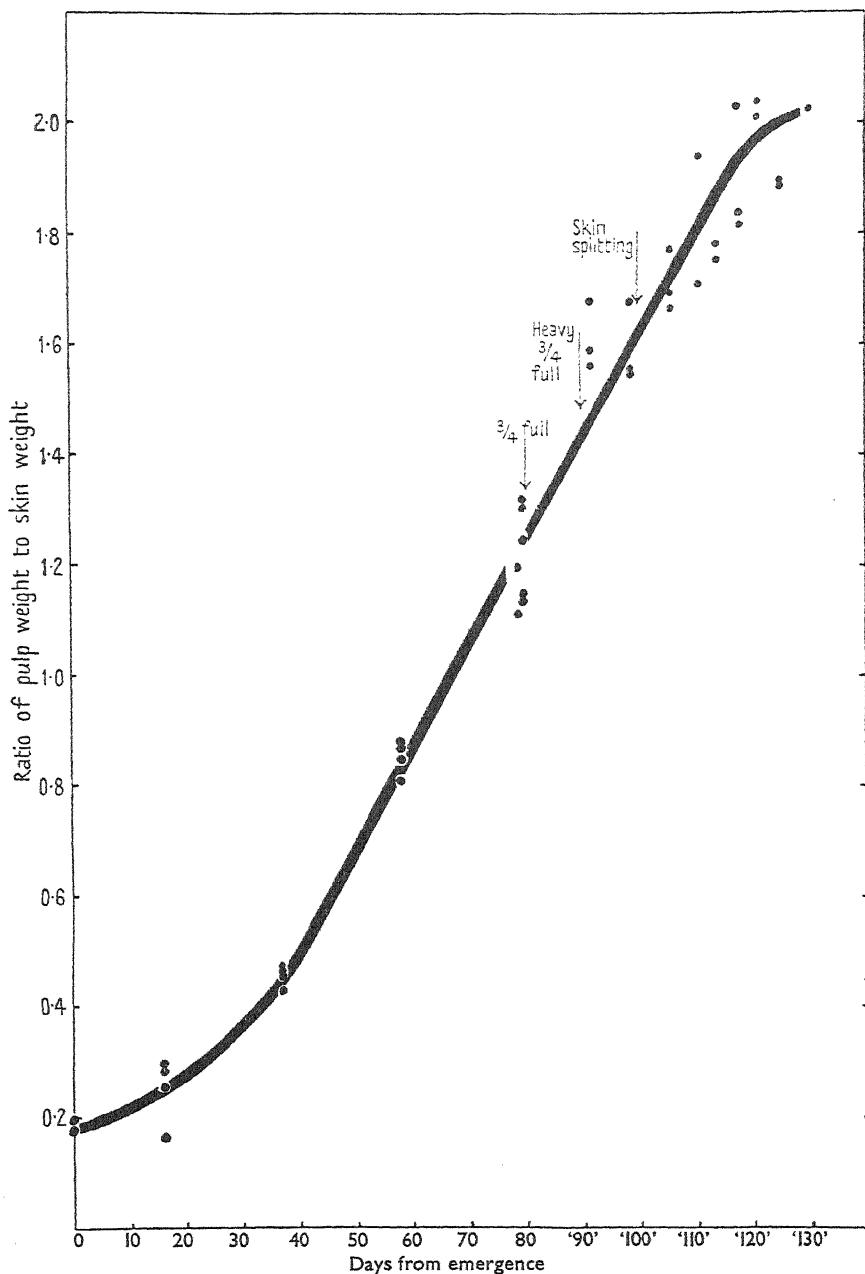


FIG. 2. The ratio of the fresh weight of the pulp to the fresh weight of the skin for fingers as in Fig. 1. The smooth curve was drawn by eye through the points of the graph.

The break in the curve indicates the end of the scale of ages of the fingers in days from emergence of the bunch and the beginning of the scale based on the arbitrary age of '80' days assigned the bunches selected as having 'standard $\frac{3}{4}$ -full' English grade fruit.

during the development of the fruit. The pulp weight increased relative to the skin from the time the bunch was shot until the fruit rotted on the tree; hence the later the fruit is cut the greater the proportion of potential edible matter. At the English grade of fruit, '½-full', the ratio was approximately 1·2 and by the '100'th day of development it had attained 1·6. It rose to values higher than 2 after '100' days, but ripening changes and yellowing of the skin had taken place to some extent by then and the fruit was unsuitable for storage.

While it must be emphasized that these data were obtained from a single estate over a short period and should not be taken as applying generally, they give, nevertheless, a quantitative expression of the relationship of the different grades of fruit obtained from one locality. The effect of local conditions on development is well shown by the weights of fingers given in Table I for samples Nos. ED/4A and ED/4B, whose ages were 79 days, but which had apparently been produced under less favourable conditions than other fruit as their values are considerably below the five individual values of the '80' day fruit and the level of the curve in Fig. 1 for this age.

TABLE I
The Development of the Banana Fruit on the Tree

(1)	(2)	(3)	(4)	(5)	(6)	(7)
Sample no.	Date of tagging, 1937.	Age on sampling, days.	Fr. wt. whole finger, gm.	Fr. wt. pulp, gm.	Fr. wt. skin, gm.	P/S ratio.
D/1A	July 28	0	8·8	1·3	7·5	0·17
1B	"	0	9·1	1·5	7·6	0·20
2A	July 12	16	43·8	9·1	34·7	0·26
2B	"	16	42·2	9·7	32·5	0·30
3A	June 21	37	69·1	21·7	47·4	0·46
3B	"	37	70·4	21·0	49·4	0·43
4A	May 31	58	93·5	41·8	51·7	0·81
4B	"	58	95·5	44·6	50·9	0·88
5A	—	80 approx.	137·9	78·0	59·9	1·30
5B	—	80 "	144·0	81·9	62·1	1·32
ED/1A	Aug. 2	16	32·7	6·7	26·0	0·26
1B	"	16	30·8	6·8	24·0	0·28
2A	July 12	37	67·0	21·3	45·7	0·47
2B	"	37	64·7	20·8	43·9	0·47
3A	June 21	58	105·7	49·2	56·5	0·87
3B	"	58	101·7	46·6	55·1	0·85
4A	May 31	79	95·8	50·5	45·3	1·11
4B	"	79	90·1	49·1	41·0	1·20

Table I (cont.)—

(1)	(2)	(3)	(4)	(5)	(6)	(7)
Sample no.	Date of tagging, 1937.	Age on sampling, days.	Fr. wt. whole finger, gm.	Fr. wt. pulp, gm.	Fr. wt skin, gm.	P/S ratio.
DT/A ₁	—	80 approx.	140.7	78.1	62.6	1.25
B ₁	—	80 "	131.6	70.9	60.7	1.17
C ₁	—	80 "	129.7	74.8	54.9	1.36
A ₂	—	'92'	167.7	103.1	64.6	1.60
B ₂	—	'92'	166.3	101.3	65.0	1.56
C ₂	—	'92'	118.1	74.0	44.1	1.68
A ₃	—	'99'	212.2	129.0	83.2	1.55
B ₃	—	'99'	177.4	107.9	69.5	1.55
C ₃	—	'99'	159.1	99.6	59.4	1.68
A ₄	—	'106'	241.6	154.5	87.1	1.77
B ₄	—	'106'	245.3	153.2	92.1	1.66
C ₄	—	'106'	162.6	102.5	60.5	1.69
A ₅	—	'111'	256.9	169.5	87.4	1.94
B ₅	—	'111'	255.8	161.3	94.5	1.71
A ₆	—	'114'	228.0	145.1	82.9	1.75
B ₆	—	'114'	271.2	173.7	97.5	1.78
A ₇	—	'118'	257.0	165.8	91.2	1.82
B ₇ (i)	—	'118'	262.5	170.0	92.5	1.84
*B ₇ (ii)	—	'118'	286.9	192.9	94.0	2.05
A ₈	—	'121'	275.0	184.1	90.9	2.03
B ₈	—	'121'	283.7	189.4	94.3	2.01
A ₉	—	'125'	322.7	211.4	111.3	1.90
B ₉	—	'125'	231.2	151.0	80.2	1.88

The fresh weights of single fingers, pulp, and skins respectively are given in columns 4, 5, and 6, for the ages shown in column 3 (i.e. days from the time of emerging of the bunches). The dates of tagging of emergent bunches are given in column 2. Column 7 gives the values of the pulp/skin ratio obtained from columns 5 and 6.

The first two sections of the table are supplied by the (a) series data (see text) in which tagged bunches were used, and the third section is obtained from the (b) series in which the arbitrary age of '80' days was assigned the three plants A, B, and C selected as bearing bunches of standard English grade '4-full' fruit.

Notes on the fruit. After '99' days splitting occurred in the skins of some of the fingers of plants A and B though these fingers remained hard and green. Plant C fell down after '106' days. Fruits on plants A and B remained green though with increasing numbers splitting until '118' days when colour-changes to yellow-green were observed mainly in the proximal hands. Fruits after this stage split and yellowed unequally through the bunch; many became brown, black, and rotted on the bunches; and many fell off each time the protecting bag was removed.

* Sample DT/B₇ (ii) had started to ripen and was yellowing.

V. DATA FROM STANDARD BUNCHES

In order to obtain data on the variability between the individual 'fingers' in a hand and the hands in a bunch, a 'nine-hand' bunch selected as English

¹ The small fingers of the Xth hand which are included in the data of Figs. 3, 4, and 5, would normally be neglected in making the commercial count.

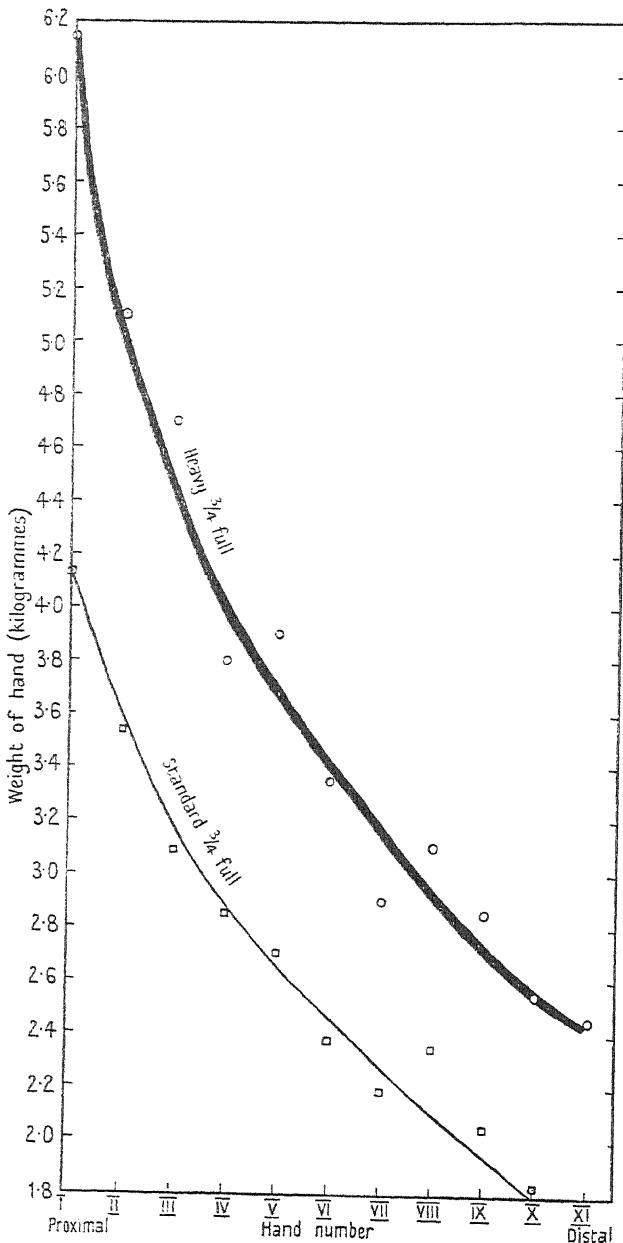


FIG. 3. Weights of individual hands of a nine-hand 'standard $\frac{2}{3}$ -full' bunch of Gros Michel bananas (squares), and of an eleven-hand 'heavy $\frac{2}{3}$ -full' bunch (circles).

(' $\frac{3}{4}$ -full') and an 'eleven-hand' Canadian grade ('heavy $\frac{3}{4}$ -full') bunch were weighed intact and then subdivided into hands by cutting across the stem medianly between hands. The hands were weighed and the fingers detached and weighed in the order in which they were attached. The skin was then removed and weighed, the pulp weight being obtained by difference. The weights of the two bunches were 28 and 42 kilograms respectively.

Fig. 3 gives the curve of distribution of weight of the different 'hands'. The similarity between the two curves indicates that the relative growth rate in all hands is maintained throughout development. The ratio (total weight of fingers)/(weight of stem piece to which they are attached) is given in Table II for the hands from the two bunches.

The ratios show irregularities due to differences in cutting the stem between successive hands, but the trend of increasing ratio from the proximal to the distal hand is evident and also the decrease in ratio as between corresponding hands in the two bunches. This indicates that, except for the proximal and second hands in both bunches where the ratio is approximately the same, the stem weight increases relative to the fingers during development.

The data for the finger weights of the standard ' $\frac{3}{4}$ -full' bunch are shown in Fig 4, white columns representing the weights of upper (proximal) and black those of lower (distal) row fingers. This gives an impression of the well marked symmetry of the Gros Michel bunch whose cylindrical shape

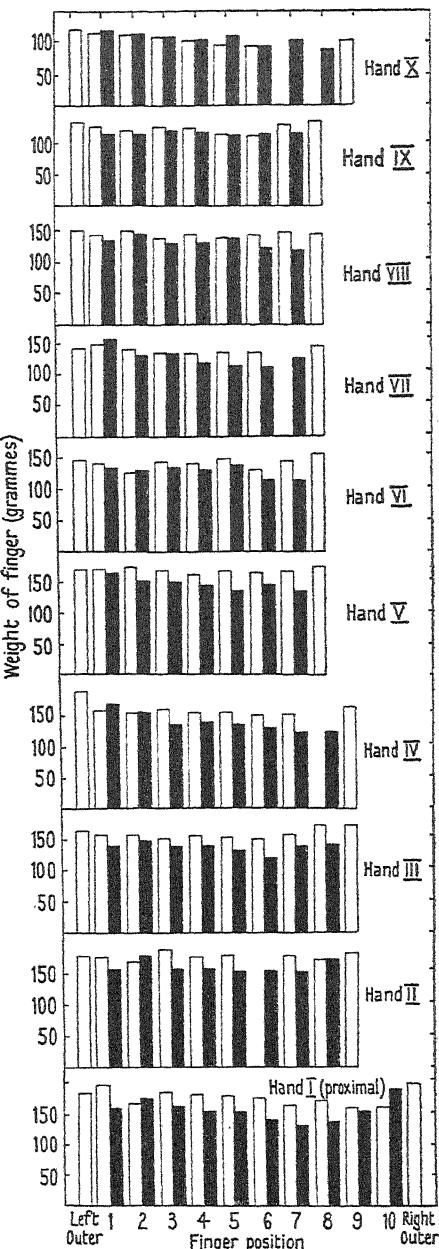


FIG. 4. Weights of individual fingers of a green, 'standard $\frac{3}{4}$ -full' bunch of Gros Michel bananas, white columns upper row, black columns lower row.

is of considerable importance in commercial handling and permits shipment of 'naked' bunches in bulk. Similar data are given for the second to the sixth hands of the 'heavy $\frac{3}{4}$ -full' bunch in Fig. 5. This showed greater irregularities than the standard ' $\frac{3}{4}$ -full' bunch, seven out of the eleven hands having one

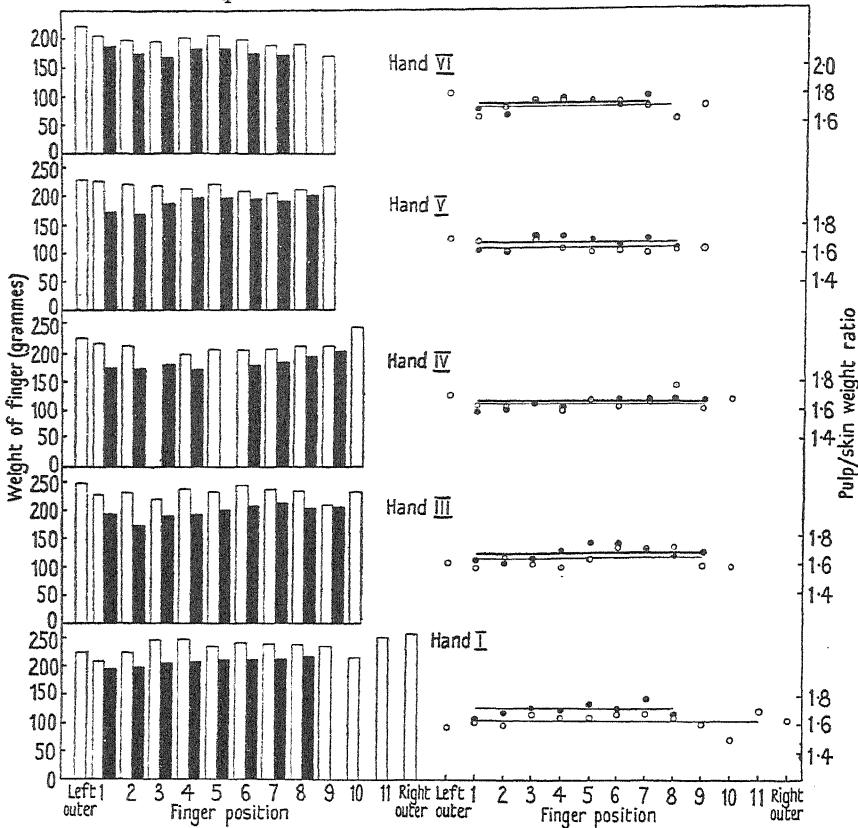


FIG. 5. Weights of individual fingers of the second to sixth hands of a 'heavy $\frac{3}{4}$ -full' bunch of green Gros Michel bananas, white columns upper row, black columns lower row, and of their pulp/skin weight ratio, white circles upper row, black circles lower row. Thin and heavy lines give mean values of pulp/skin ratio for upper and lower row fingers in each hand, omitting the two outer fingers.

or more fingers missing from the usual pairs in top and bottom row. The pulp/skin weight ratios for the fingers of the different hands are shown alongside, white circles representing the ratios of upper and black those of lower row fingers, the mean values being given as thin and heavy lines respectively. The high degree of uniformity in finger weight and in pulp/skin weight ratio in each row of each hand is apparent. The weight of fingers in the upper row is almost invariably greater than that of the corresponding lower row. The pulp/skin weight ratio is consistently greater in the lower

than the upper row, even for fingers of approximately equal weight in the two rows.

TABLE II
Ratio of Finger Weight to Stem Weight

Hand.	'Standard $\frac{3}{4}$ -full' bunch.	'Heavy $\frac{3}{4}$ -full' bunch.
I	10.09	11.40
II	15.07	14.49
III	19.46	15.62
IV	21.26	15.28
V	20.61	19.14
VI	22.54	16.94
VII	22.45	17.21
VIII	28.49	21.89
IX	28.70	19.92
X	24.97	27.85
XI	—	13.66*

* Stem weight included a portion bearing hermaphrodite flowers and ratio is therefore lower than for the other hands.

TABLE III

Mean Weight (gm.) of Fingers and Mean Pulp/Skin Ratio

	I.	II.	III.	IV.	V.	VI.	VII.	VIII.	IX.	X.	XI.	
Mean wt.	Upper row	236.2	233.3	229.6	208.6	217.0	197.8	179.8	182.8	166.4	161.7	141.6
	Lower row	205.4	206.2	197.3	181.1	188.9	178.2	159.0	164.0	154.6	146.6	137.6
No. of fingers	25	21	20	18	18	17	16	17	17	17	16	
	Upper row	1.68	1.62	1.66	1.65	1.64	1.72	1.60	1.60	1.56	1.48	1.46
Mean pulp/skin ratio	Lower row	1.74	1.71	1.70	1.64	1.67	1.74	1.63	1.63	1.61	1.54	1.50

Mean weight of finger in bunch = 191.3 gm.

Mean pulp/skin ratio " = 1.65 gm.

Table III and Fig. 6 give the mean weight of fingers and the mean pulp/skin weight ratio in the upper and lower rows of the hands of the 'heavy $\frac{3}{4}$ -full' bunch. The two outermost fingers in each hand are usually considerably above average size and more curved; they have been omitted in calculating the mean value. It will be seen that (1) there is a fairly regular decrease in weight of fingers from proximal to distal hands, whereas (2) the pulp/skin ratios show fairly steady values from the proximal to the VIth or VIIth hands and then a regular decline in the smaller hands; (3) fingers of approximately the same weight as the mean for the bunch occur in the upper row of hand VI and the lower of hand V; (4) fingers with the mean pulp/skin ratio for the bunch occur in the upper row of hands I to VI and the lower row of hands IV to VIII.

As a further method of expression of the uniformity of the material within a bunch the coefficients of variability of the finger weight in the whole bunch and for the upper and lower rows of the different hands have been calculated and are given in Table IV.

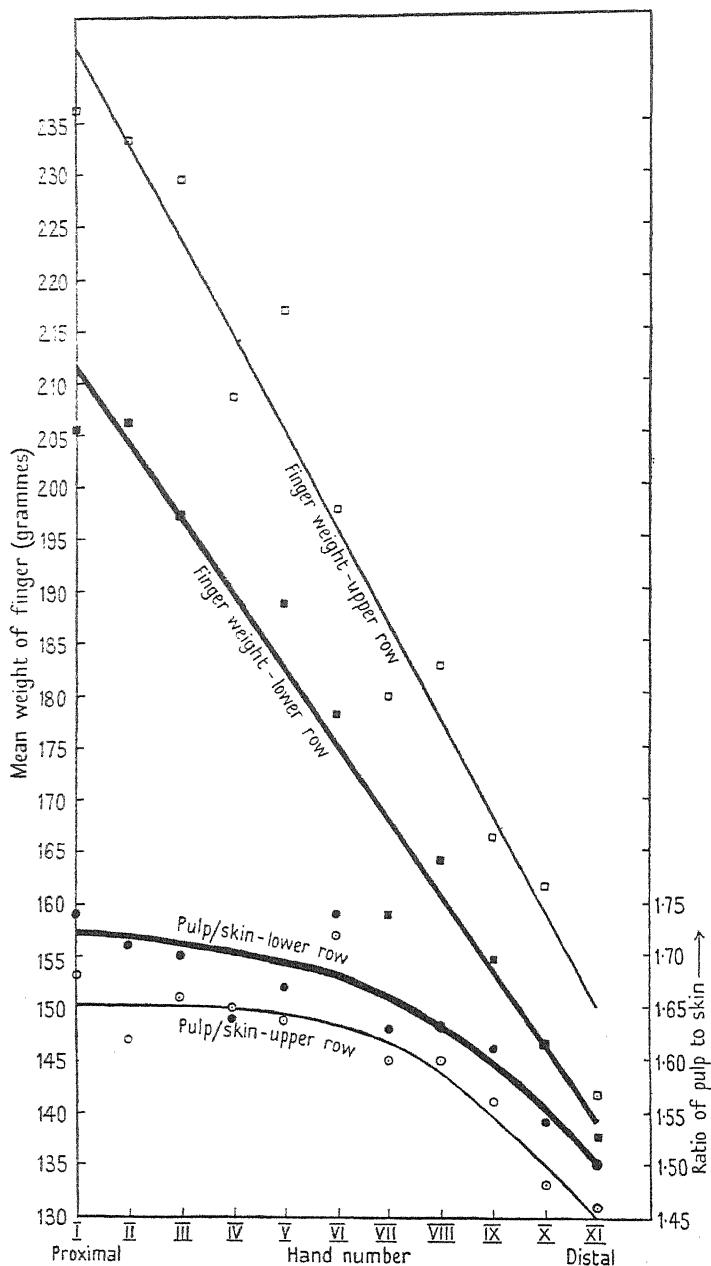


FIG. 6. Mean weight of fingers in upper (white squares) and lower (black squares) rows of each hand (excluding the two outer fingers in each hand) of an eleven-hand 'heavy $\frac{2}{3}$ -full' bunch of Gros Michel bananas, and mean pulp/skin weight ratio for the same fingers (white circles upper, black circles lower, rows).

TABLE IV

Coefficients of Variability of Finger Weight within Rows

Hand	I.	II.	III.	IV.	V.	VI.	VII.	VIII.	IX.	X.	XI.
Upper .	. 3·16	5·67	4·45	2·93	3·13	4·27	5·30	2·41	5·53	4·68	3·83
Lower .	. 4·16	3·60	6·09	6·41	6·31	3·90	8·98	3·13	5·34	3·85	3·45

The mean coefficient of variability for fingers from the upper rows is 4·12 per cent., from lower rows 5·20 per cent., and for both rows 4·66 per cent., whilst the coefficient for a single observation for a finger from the whole bunch is 16·96 per cent. These values are relatively low for biological material: the individual finger weights are not entirely independent and the variability within rows is thus to some extent reduced. The same qualifications as to the general applicability of the data on these two commercial bunches apply as in section IV.

The surface/volume ratio is important in many metabolic phenomena whose measurement is undertaken in detached plant organs, e.g. transpiration, respiration, and heat liberation. Whilst no values have so far been obtained directly for this ratio itself in bananas it may be assumed, until disproved, that the density of the pulp and skin and the thickness of skin are the same for fingers in both rows. Lower row fingers have a higher pulp/skin weight ratio than upper row fingers, even for fruit of approximately the same weight: they should, therefore, on the above assumption, have lower surface/volume ratio.

Whenever closely comparable fingers are required for experimental work they have, for the reasons given above, been selected (1) from hands between the second and sixth, (2) from inside fingers rather than the two outer ones in any individual hand, and (3) from the same row in different hands rather than from the upper and lower rows in the same hand.

Smith (1932) states that there is considerable variation in the ratio of pulp to skin, between bananas in a bunch, and even within a single hand. He adds that the main difference is between the relatively straight bananas on the inside of the hand and the more strongly curved ones on the outside. The inside straighter bananas have relatively more skin and less pulp. It may be noted that the inside fingers with which he was dealing weighed only 90 to 95 grams and had a pulp/skin ratio of 1·0: this corresponds to somewhat less than $\frac{3}{4}$ -full' fruit on the basis of the material considered here (Figs. 1 and 2).

VI. SUMMARY

1. A brief description is given of the development and morphology of the banana, which exhibits considerable differences from other fruits.
2. Some of the causes of variability in the final size of the fruit bunch are discussed in relation to the subsequent behaviour during storage.

3. For the Gros Michel banana data are given of weights of individual 'fingers' and of the proportion of pulp to skin during development up to the final senescence and collapse of the individual plant.
4. In relation to the choice of experimental material, corresponding data are given for individual fingers and 'hands' of bunches at the stages of maturity adopted in commercial practice for harvesting with subsequent cold storage.

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Gametogenesis and Embryogeny of *Tamarix ericoides* Rottl.¹

BY

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With forty-two Figures in the Text

INTRODUCTION

LITERATURE on the Tamaricaceae reveals information only on *Myricaria germanica* and a few European and an Indian species of *Tamarix*. Frisendahl (1912) described the development of *Myricaria germanica*, noting that it corresponds to the Adoxa type, but with many modifications. This has been confirmed by the work of Zabban (1936) on the same species of *Myricaria*. Mauritzon (1936) working on *Tamarix tetrandra* and five other species of the same genus reports the Adoxa type of development of the embryo-sac. Joshi and Kajale (1936), working on *Tamarix dioica* (a dioecious species), and Puri (1937) on *Tamarix chinensis*, state that the development of the embryo-sac is of the Fritillaria type. Joshi and Kajale further state that since there were no male plants in the neighbourhood of the female plants of *Tamarix dioica*, fertilization and embryogeny do not occur; instead, the embryo-sacs degenerate, and seeds containing no embryos in them are dispersed. With a view to comparing the life-histories of these species of *Tamarix* with *Tamarix ericoides*, the present investigation was taken up.

MATERIAL AND METHODS

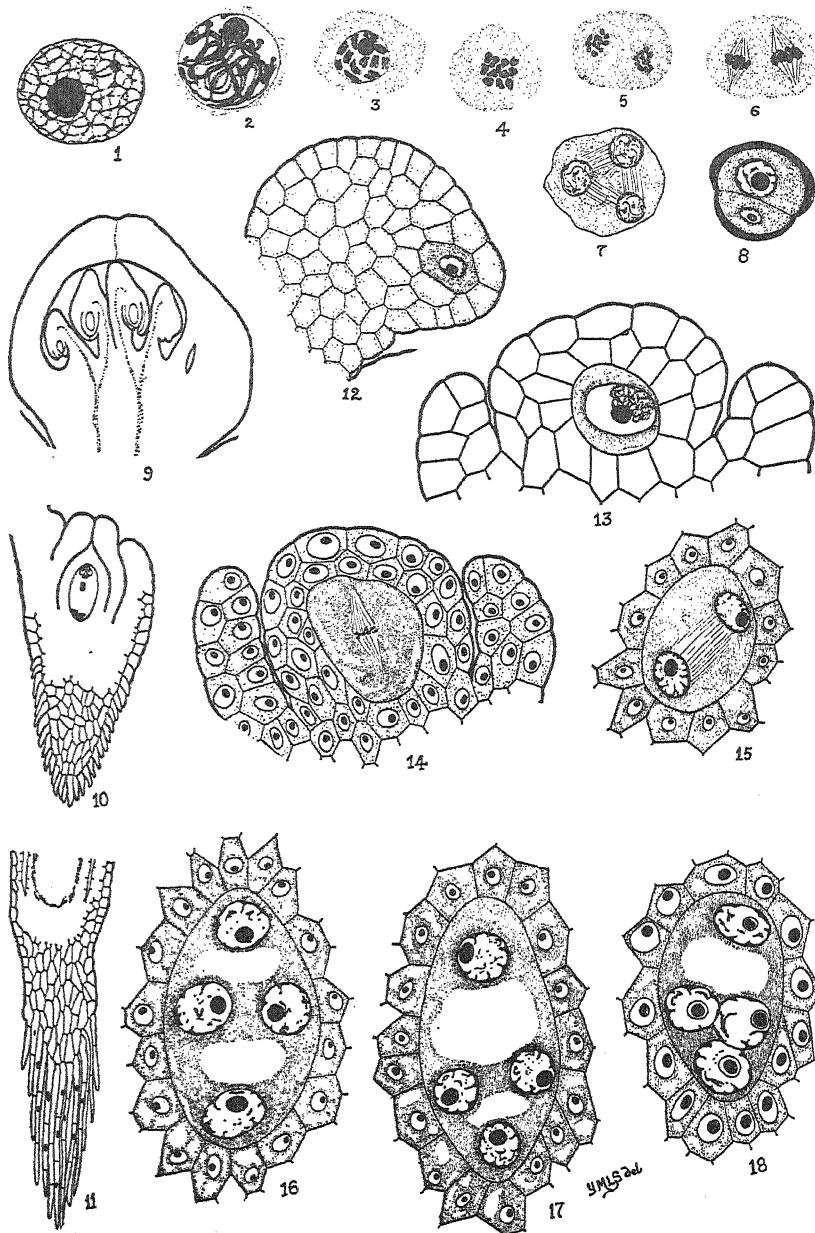
Tamarix ericoides Rottl. grows gregariously in large patches in the bed of the river Kanva at Chennapatna (about 39 miles from Bangalore, Mysore State), and displays an ericoid habit resembling closely *Myricaria germanica*, its allied genus. The material was collected in November 1937, and fixed in Bouin's fluid. Sections were cut ranging from 8 to 16 microns in thickness and stained in Heidenhain's iron-alum-haematoxylin.

MICROSPOROGENESIS

In the development of the anther the tapetum is formed by the outermost layer of the sporogenous tissue. At the tetrad stage of the microspores, the

¹ Part I of the thesis approved for the degree of Master of Science of the University of Mysore, 1938.

[Annals of Botany, N.S. Vol. III, No. 12, October 1939.]



Figs. 1-18. Fig. 1. Prophase in the nucleus of the microspore mother-cell. $\times 900$. Fig. 2. Pachytene threads. $\times 600$. Fig. 3. Diakinesis showing twelve bivalents. $\times 450$. Fig. 4. Polar view of the metaphase plate showing twelve haploid chromosomes. $\times 450$. Figs. 5 and 6. Second division. $\times 450$. Fig. 7. Tripolar spindle and the formation of the tetrad of micro-

tapetal cells are large, binucleate, and rich in their contents, but later on they disorganize to afford nutrition to the developing tetrads of microspores.

The reticulum of the nucleus in the microspore mother-cell resolves itself into a number of pachytene threads, the double nature of which is visible only in the region of the loops. At diakinesis twelve bivalents can be counted (Figs. 1-3). The chromosomes condense greatly and get deeply stained. With the disappearance of the nuclear membrane and the formation of the spindle-fibres, the chromosomes are separated and drawn apart to the poles on the spindle.

The second division is homoeotypic and the tetrads separate by cleavage furrows. The mature pollen grain has a large tube nucleus and a smaller generative cell. The pollen grain is covered by a thin intine and a thick exine (Figs. 4-8). During the study of meiosis in the microspore mother-cells the number of bivalent chromosomes was determined to be twelve.

MEGASPOROGENESIS

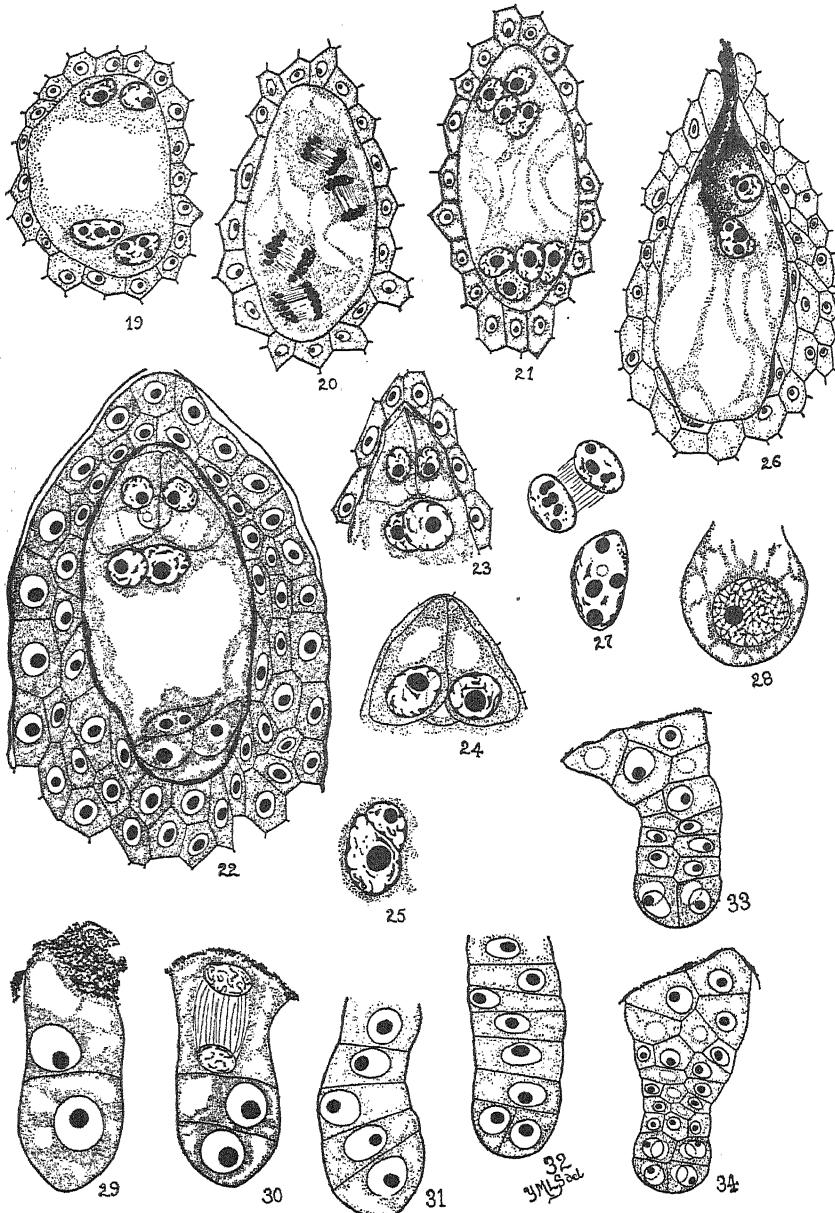
The ovary is superior, tricarpellary, and trilocular, with a single style and free stigmatic lobes. The ovules are indefinite in number. Placentation is basal and the ovules are anatropous, their micropyles pointing towards the placental ridge (Fig. 9). A vascular trace is given off to each ovule through the funiculus.

The ovules have two integuments. The initials of the integuments appear after the formation of the archesporial cell. The integuments grow, and in the mature ovule the inner one projects beyond the outer and alone forms the micropyle. The behaviour of the epidermal cells at the chalazal end of the ovule is very characteristic. They grow out after fertilization and develop into a tuft of long, multicellular, and uniseriate hairs which play an important part in the dispersal of the seeds (Figs. 10 and 11).

The primary archesporium, consisting usually of a single cell and rarely of two, is hypodermal in origin (Fig. 12). The archesporial cell cuts off a parietal cell unlike that of *Myricaria germanica* (Frisendahl, 1912). The formation of a parietal cell has been observed in other species of *Tamarix* also.

The megasporangium, which is at first polyhedral in outline, becomes rounded off as it enters into the meiotic division. Without the formation of a tetrad, the megasporangium functions directly as a megasporangium. The first meiotic division in the nucleus of the megasporangium results in

spores. $\times 450$. Fig. 8. Mature pollen grain showing the large tube nucleus and the smaller generative cell. $\times 900$. Fig. 9. Longitudinal section of a young ovary to show the basal placentation and the anatropous nature of the ovules. $\times 19$. Figs. 10 and 11. Showing the gradual elongation of the epidermal cells at the chalazal end of the ovule. $\times 40$ and $\times 27$. Fig. 12. Archesporial cell before cutting off a parietal cell. $\times 300$. Figs. 13 and 14. Meiosis in the megasporangium. $\times 300$. Fig. 15. Two-nucleate embryo-sac. $\times 450$. Fig. 16. Four-nucleate embryo-sac with its nuclei arranged in the cruciform manner. $\times 450$. Figs. 17 and 18. Stages in the formation of the 1+3 arrangement of the nuclei, in the four-nucleate embryo-sac. $\times 450$.



Figs. 19-34. Fig. 19. Secondary four-nucleate embryo-sac. $\times 300$. Fig. 20. Formation of eight-nucleate embryo-sac from the secondary four-nucleate stage. $\times 300$. Fig. 21. Eight-nucleate embryo-sac before organization. $\times 200$. Fig. 22. Organized eight-nucleate embryo-sac. $\times 300$. Fig. 23. Egg apparatus with beaked synergids and with the two polars. $\times 300$. Fig. 24. Egg apparatus showing the 'egg-like' synergids. $\times 450$. Fig. 25. Two polar nuclei

two nuclei, which migrate to the poles of the megasporangium, rendering it an appearance of the embryo-sac with two nuclei (Figs. 13, 14, and 15).

A four-nucleate embryo-sac is later formed as a result of the division of the two nuclei. In most cases the four nuclei are arranged in a cruciform or $1+2+1$ manner (Fig. 16). Occasionally one nucleus is seen to remain at the micropylar end, while three others migrate to the chalazal end. Later the two nuclei present in the centre of the embryo-sac also migrate to the bottom, forming the $1+3$ arrangement. Thus the $1+3$ arrangement often seen also seems to be derived from the cruciform type (Figs. 16, 17, and 18).

A four-nucleate embryo-sac is also seen with two small nuclei at the micropylar end and two large nuclei at the chalazal end of the embryo-sac (Fig. 19). The micropylar nuclei have each one nucleolus, while the chalazal nuclei have mostly three nucleoli in each of them, indicating their haploid and triploid nature respectively, just as Bambacioni (1927) has shown in *Fritillaria persica*, and Joshi and Kajale in *Tamarix dioica* (1936). This is the secondary four-nucleate stage of the embryo-sac (the author is at work to study the cytological details between these two stages).

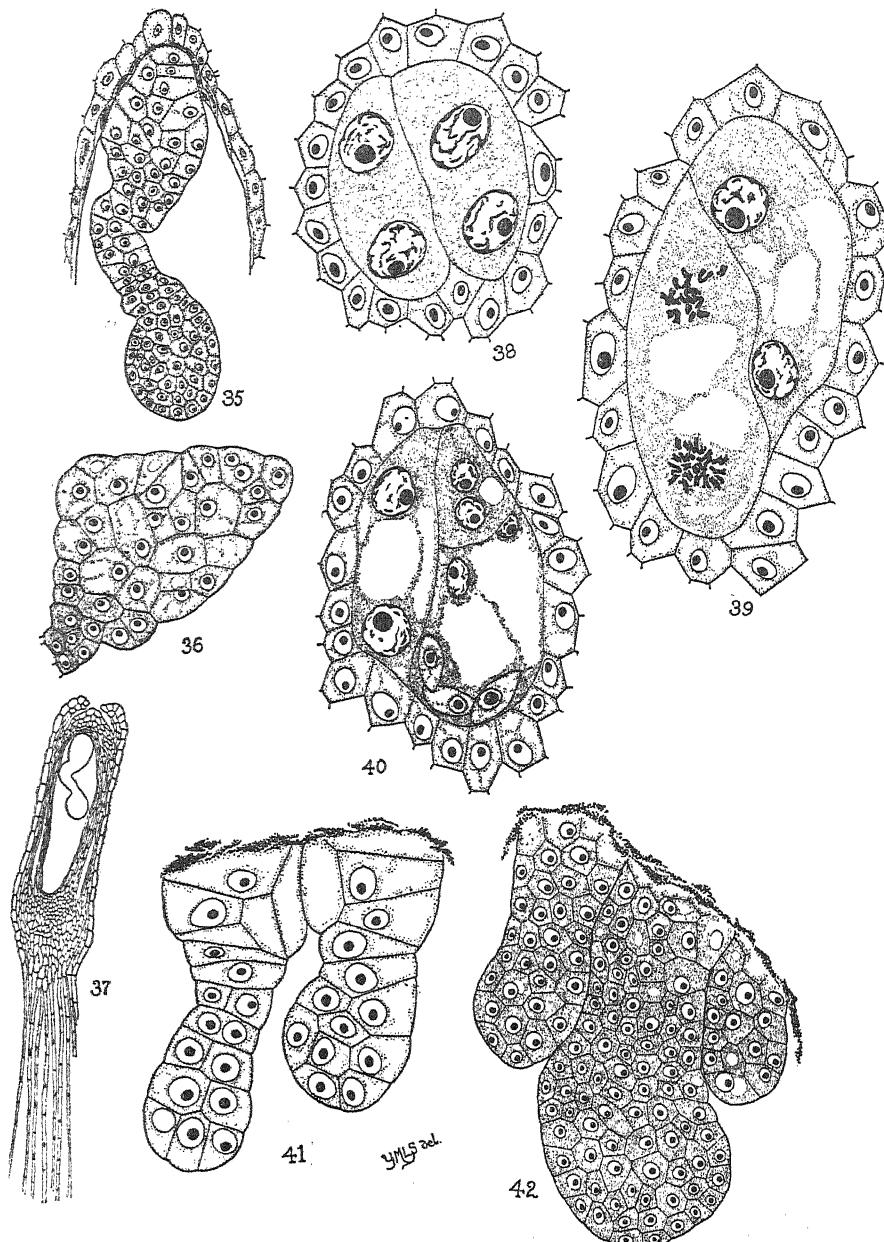
All the four nuclei divide simultaneously. The number of chromosomes seen on the telophase spindles at the chalazal end is greater than that at the micropylar end (Fig. 20). This is a further evidence to show the haploid and triploid nature of the micropylar and chalazal nuclei respectively. The result of this division is the eight-nucleate embryo-sac, where the nuclei of the chalazal set are larger than those of the micropylar set (Fig. 21).

These eight nuclei organize into the cells of the embryo-sac, namely, the egg apparatus, antipodals, and two polar nuclei (Fig. 22). The synergids are beaked (Fig. 23). Very rarely the synergids show a peculiar vacuolation which is egg-like. The vacuoles of the synergids are situated towards the micropylar end and give them the appearance of the 'egg' (Fig. 24). The antipodal polar is usually bigger than the micropylar polar and corresponds in its size to that of the antipodals (Fig. 25). The polars press against each other but do not fuse, and lie close to the egg apparatus, as has been observed by Frisendahl (1912) in *Myricaria germanica* (Fig. 23). The antipodals, which are at first small, usually develop vacuoles and grow larger in size. They are inconsistent in their behaviour, just as Mauritzon (1936) reports in *Tamarix tetrandra* and other species. The enlargement of the embryo-sac is accompanied by the disorganization of one or two layers of the nucellus lining the embryo-sac.

FERTILIZATION AND ENDOSPERM FORMATION

The pollen tube enters the embryo-sac by the micropylar end, and fertilization is normal (Fig. 26). The primary endosperm nucleus divides

lying close to each other; note the large size of the chalazal polar. $\times 450$. Fig. 26. Fertilization. $\times 133$. Fig. 27. Endosperm nuclei; note the presence of five nucleoli showing the pentaploid nature. $\times 300$. Fig. 28. Fertilized egg. $\times 300$. Figs. 29, 30, and 31. Linear pro-embryos. $\times 300$. Fig. 32. Pro-embryo of seven cells with vertical division in the distal cell. $\times 210$. Figs. 33 and 34. Quadrant and octant stages of the embryo. $\times 210$.



Figs. 35-42. Fig. 35. Fully developed embryo with the massive suspensor and the large pad of tissue developed from the proximal cell. $\times 93$. Fig. 36. The tissue developed from the proximal cell of an embryo. $\times 300$. Fig. 37. Ovule showing the large size of the embryo in it. $\times 150$. Fig. 38. Double two-nucleate embryo-sacs. $\times 300$. Fig. 39. Double embryo-

prior to the division of the fertilized egg. The endosperm nuclei are large and elliptical, containing five nucleoli in the earlier stages and a single large nucleolus later, which is formed from the fusion of the five nucleoli (Fig. 27). The endosperm nuclei are pentaploid and ten to fifteen in number. The cellular endosperm that Mauritzon (1936) reports in *Tamarix tetrandra* does not form here.

EMBRYO

The fertilized egg undergoes a period of rest and divides only later when two or four endosperm nuclei have been formed. The first division is transverse. Some more transverse divisions occur resulting in a linear pro-embryo of six to seven cells (Figs. 28-32). The distal cell undergoes a vertical division and later a transverse division, giving rise to the quadrant stage. The next stage is the octant, which by further divisions gives rise to a bulbous embryo like the typical dicotyledonous one (Figs. 33-5).

The proximal and suspensor cells undergo important changes. Vertical divisions occur in all the cells of the suspensor after the formation of a vertical wall in the distal cell. The result of these and some more divisions is the formation of a massive suspensor. The behaviour of the proximal cell is interesting. It undergoes a number of vertical and transverse divisions in the beginning, and later oblique divisions, giving rise to a large massive pad of cellular tissue, completely filling the cavity of the embryo-sac at its micropylar end. This tissue developed by the proximal cell together with the suspensor will be much larger than the bulbous portion of the embryo (Figs. 35 and 36).

The nucellar cells elongate to a large extent, forming a loose thin-walled perisperm around the cavity of the embryo-sac containing the embryo. The cavity of the embryo-sac itself becomes greatly elongated and the embryo occupies nearly half to two-thirds of its length (Fig. 37). The ovary also increases in size and develops into the fruits, ultimately dehiscing by three valves, dispersing the plumed seeds containing the embryos.

ABNORMALITIES

Occasionally ovules with double embryo-sacs have also been observed. The juxtaposition of the two embryo-sacs suggests that they must have been derived from two archesporial cells situated side by side (Figs. 38, 39, and 40). Instances of polyembryony are also noticed. Two embryos in one ovule and three in another are seen. In the three-embryo case the central one is larger than the two laterals, and all the three appear so massive that it is hard

sacs, one of them which is in the 1+3 stage dividing. $\times 450$. Fig. 40. Double embryo-sacs, one of them being fully developed. $\times 300$. Fig. 41. Abnormal case of double embryos developing in the same embryo-sac. $\times 300$. Fig. 42. An abnormal case of three embryos developing in the same embryo-sac. $\times 200$.

to distinguish the suspensor or the proximal cell tissue from the embryo proper (Figs. 41 and 42).

CONCLUSIONS

The development of the embryo-sac in *Tamarix ericoides* is of the Fritillaria type. Frisendahl (1912) described the development of *Myricaria germanica*, noting that it corresponds to the Adoxa type, but with many modifications. He has often observed both the chalazal nuclei in the four-nucleate stage increase in size and number of chromosomes; mature embryo-sac with only one nucleus at the micropylar end separated by a large vacuole from three nuclei at the chalazal end. These observations have been recently reinterpreted by Schnarf (1931) in the light of Bambacioni's work on the embryology of *Fritillaria persica*, stating that the embryo-sac with a 1+3 arrangement of the nuclei is formed, and that during the next division the three chalazal nuclei fuse while passing through the metaphase, anaphase, and telophase stages, and that out of the original four-nucleate embryo-sac, again a four-nucleate embryo-sac results having two small haploid nuclei at the micropylar end and two large triploid nuclei at the chalazal end. Zabban (1936) working on *Myricaria germanica* has further confirmed this statement, while his figures show the details of the nuclear changes between the 1+3 stage and the secondary four-nucleate stage of the embryo-sac.

Bambacioni and Giombini (1930) described the same phenomenon in *Tulipa gesneriana*, and later, Bambacioni by herself (1931) has made similar observations in *Tulipa praecox* and *Lilium bulbiferum*. Cooper (1935) investigating on a number of species of *Lilium* finds the Fritillaria type in all. Romanov (1936) reports the same type of development in *Gagea Ova* and *Gagea grammifolia*, and considers its occurrence to be very probable in *Gagea tenera* also, which was not so fully studied. By comparing Stenar's (1927) figures of *Gagea lutea* with his own preparations of these three species, he comes to the conclusion that the same type occurs in Stenar's plant also.

The embryo-sacs in all these cases are tetrasporic, where the egg is removed from the megasporangium mother-cell by four divisions. The result of the four divisions is a sixteen-nucleate embryo-sac. But this does not correspond with the sixteen-nucleate embryo-sac of *Peperomia*, because, normally, it does not look like that. This normal eight-nucleate appearance of the sixteen-nucleate embryo-sac is brought about by the interpolation of a secondary four-nucleate embryo-sac.

Mauritzon (1936) investigating on *Tamarix tetrandra* and five other species of the same genus (*T. aestivalis*, *T. africana*, *T. gallica*, *T. odessana*, and *T. pentandra*) reports the Adoxa type in all. Joshi and Kajale (1936) report in *Tamarix dioica* the Fritillaria type of development. Puri (1937) also reports the Fritillaria type in *Tamarix chinensis*. Maheshwari (1937) reviewing observes, 'Since Mauritzon's figures do not give any indication of a 1+3 arrangement, nor a difference in the sizes of the nuclei at the two ends of the

embryo-sac, it is not possible to go further into the question, and a fuller investigation of the genus *Tamarix* seems to be desirable.' The present observations lead to the conclusion that the development of the embryo-sac of *Tamarix ericoides* Rottl. is of the Fritillaria type.

Beaked synergids occur in a large number of plants. But the peculiar 'egg-like' vacuolation of the synergids is very characteristic and rare among the angiosperms. Frisendahl (1912) observed such synergids in *Myricaria germanica*, Osterwalder (1898) in *Aconitum napellus*, Persidsky (1914) in *Delphinium elatum*, Puri (1934) in *Moringa oleifera*, Joshi and Rama Rao (1936) in *Gisekia pharnaceoides*, and Joshi and Kajale (1936) in *Tamarix dioica*.

SUMMARY

1. The haploid chromosome number in *Tamarix ericoides* Rottl., reported for the first time, is twelve.
2. The archesporial cell is single, rarely double.
3. There is no tetrad formation, division of the megasporangium mother-cell; instead, a four nucleate embryo-sac results.
4. The nuclei are originally arranged in a cruciform type, but later form a $1+3$ arrangement.
5. As a result of the third division, the embryo-sac is again four-nucleate, with two haploid nuclei at the micropylar end and two triploid nuclei at the chalazal end.
6. The cells of the embryo-sac are formed at the end of the fourth division, the embryo-sac thus representing the sixteen nucleate condition.
7. The development of the embryo-sac of *Tamarix ericoides* conforms to the Fritillaria-type.
8. The endosperm is free nuclear, and the embryo is characteristic in developing a massive suspensor and a large pad of cellular tissue from the proximal cell.
9. Double embryo-sacs and the occurrence of polyembryony are reported for the first time in the family.

ACKNOWLEDGEMENTS

I am indebted to Dr. M. A. Sampathkumaran, M.A., Ph.D., University Professor of Botany, Mysore University, for suggesting the problem and guidance throughout the course of this work. Sincere thanks are due to Professor L. Narayana Rao, M.Sc., F.R.M.S., and other members of the staff for discussion and helpful suggestions. I thank Dr. K. P. Biswas, Director, Royal Botanic Gardens, Sibpore, Calcutta, for confirming the identification as *Tamarix ericoides* Rottl., and Dr. P. Maheshwari for lending me some literature and helpful criticism.

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Resupination Studies of Flowers and Leaves

BY

ARTHUR W. HILL

With eight Figures in the Text

THE term 'resupination' is usually applied to the flowers of those orchids which twist through an angle of 180° in order that the labellum, which in the bud is adaxial, may become the lowermost member of the perianth and thus be adjacent to the subtending floral bract. The strict meaning of the Latin word, 'to bend or turn back', or 'lying on one's back,' does not quite accurately describe the phenomenon exhibited by the majority of the orchids and by many other flowers, as well as by certain leaves which turn round, since the torsion as a rule is not a 'bending back' but a twist either clockwise or counter-clockwise, which brings the labellum from the XII o'clock to the VI o'clock position, and is thus a 'turning round' rather than a bending or turning back.

This twisting round of flowers or rather of the flower buds is a much more common occurrence than is usually recognized; nor is it a speciality of the orchid family. When the pendent raceme of any plant bearing zygomorphic flowers is considered, for instance, it is, of course, obvious that every flower on the raceme has twisted through an angle of 180° in order that the keel—as for example in *Laburnum* (Goebel, 1920, p. 237), *Wistaria*, or *Robinia*—may be on the lower side of the flower. The younger buds on such pendent racemes, it will be noticed, are upside down, but as they develop the pedicels twist and invert the flowers, thus bringing the standard above and the keel below, as they would normally be in such flowers on a vertically erect spike or raceme, as, for instance, that of *Genista*, *Spartium*, *Coleus*, &c.

Regular flowers on pendent spikes or on upright racemes tied vertically downwards do not appear to undergo any torsion.

Torsion of the zygomorphic flowers borne on normally erect inflorescences quickly takes place when the inflorescences are bent over and tied vertically downwards—and in the course of two days the opening flowers of *Coleus Autranii*, for example, turned through an angle of 180° in order to adjust their position and place the keel on the lower side of the flower. The flowers of *Saintpaulia ionantha* (Fig. 1) behave in a similar manner, for when a flowering plant is hung upside down they will twist completely round through 180° in the course of one to two days, in the counter-clockwise direction, and when the plant is again set upright the flowers untwist and regain their normal pose. The flowers of *Genista fragrans* and *Chorizena ilicifolium* also

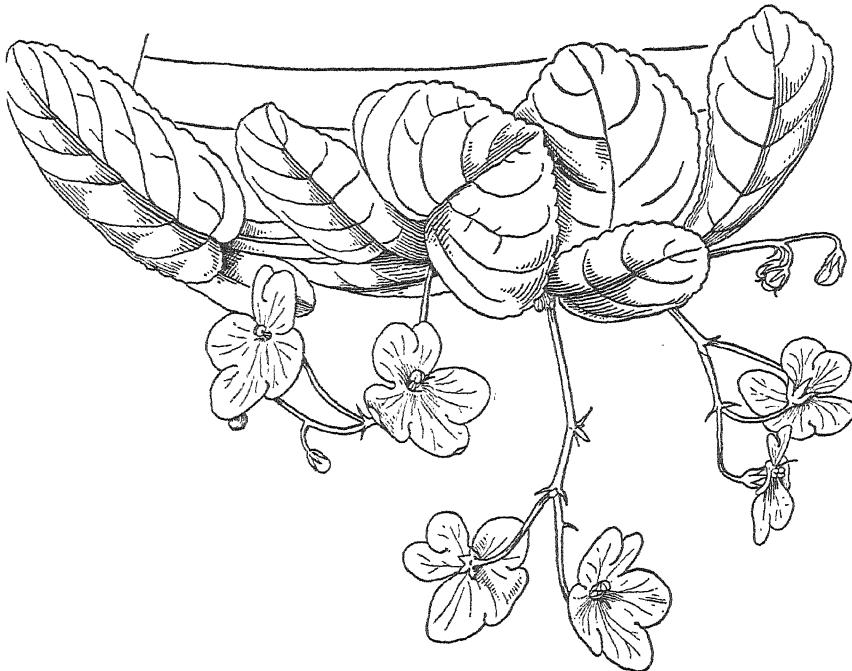


FIG. 1. *Saintpaulia ionantha* Wendl. The lower pot was grown in the normal upright position and shows the zygomorphic flowers, the two anthers lying close below the upper two corolla-lobes. The upper pot was hung in an inverted position, and in the course of two days all the flowers had twisted round. ($\times \frac{2}{3}$).

quickly invert themselves when the inflorescences are tied to point vertically downwards.

The state of affairs in those orchids which bear either 'resupinate' flowers or 'non-resupinate' flowers on pendent racemes is also a matter of interest, for in the former case the flowers must either have untwisted 180° , in order to get the labellum to the lower side of the flower, or else have twisted a further 180° , making a complete circle of 360° . The 'non-resupinate' flowers on a pendent raceme, on the other hand, like those of Laburnum, &c., will also have undergone a twist of 180° so that the labellum can regain its normal adaxial position.

An interesting instance is afforded by the flowers of *Columnea gloriosa* (Fig. 2); this plant is grown at Kew in hanging baskets and the leafy shoots hang vertically downwards. The scarlet, strongly zygomorphic flowers borne in the leaf axils are erect and face away from the stem, the peduncles being curved upwards and also slightly twisted. In consequence of the stems being pendent the flower buds have twisted round so that the hooded portion of the perianth, shielding the stamens and pistil, is uppermost and the back of the flower is next to the stem.

When one of these shoots is tied in a vertically upright position, the peduncles of the flower buds gradually bend upwards until they are once more erect. The front of the bud is then facing the stem, and it opens in this position and apparently is unable to effect the further lateral twist which would bring the flowers into the position facing away from the stem which it occupies when the stems are pendent.

Before referring briefly to some of the remarkable examples of resupination exhibited by the orchids, a few cases of normal resupination in other plants may be referred to. Among these are *Bolusia resupinata* Milne-Redhead (Leguminosae), figured in Hooker's 'Icones Plantarum' t. 3246, which has its flowers inverted through the bending back of the pedicel; *Salvia Jurisicai* Košanin, figured in the 'Botanical Magazine' (t. 9250), the peduncles of whose flowers twist through 180° as the flowers open; and *Canavalia ensiformis* DC. (Cammerloher, 1924) where, owing to the curvature of the peduncles, the flowers are inverted and the standard is on the lower side of the flower.¹ Cammerloher considers that floral resupination in this case is a developmental movement—'entfaltungsbewegung'—and is not connected with the visits of insects. The peduncles in this species, like those of *Columnea*, are negatively geotropic.

Mention should also be made of the flowers of *Brachysema lanceolatum* Meissn. (Leguminosae) (Fig. 3) since they stand vertically upright in pairs at the nodes of the flowering stems. In this species the standard (Fig. 3, s) and wings are very small and the erect, crimson-red, upright keel is the conspicuous part of the flower. In this case the flower appears to have turned through an angle of only 90° .

¹ Other species of *Canavalia* have normal non-resupinate flowers with the standard above and keel below.



S.R.C.

FIG. 2. *Columnea gloriosa* Sprague. The pendulous stems, as seen on the right-hand side, bear their flowers facing *away* from the stems. The shoot on the left was tied vertically up. The three flowers as well as the buds have all turned up, but have not succeeded in turning round to face away from the stem as they normally do. (Reduced.) Note the apogeotropic curvature of the pedicels.

The orchids present many curious and interesting problems in connexion with the phenomenon of resupination, as Prof. Oakes Ames has recently shown in his interesting paper on 'Resupination' (Ames, 1938).

The twisting of orchid flowers has long attracted the attention of botanists, and Charles Darwin (1890, p. 129) pointed out the remarkable case of *Malaxis paludosa*, the flowers of which twist through an angle of 360° in order to get the labellum in the superior position which it would have occupied had the flowers remained untwisted. The species *M. monophyllum* is further remarkable, as Oakes Ames (1938) points out, since the American specimens twist through an angle of only 180° , whereas the Old World plants of this species resemble *M. paludosa* and make the complete twist through 360° , thus bringing the labellum to its primitive or non-resupinate position.

A certain number of orchids, as is well known, have erect inflorescences with the flowers untwisted or non-resupinate—that is, the labellum is in the adaxial position—and in the same genus, as for instance *Epidendrum*, some species (*E. ciliare*, *E. nocturnum*) have resupinate flowers, while in others, such as *E. fragrans*, *E. Allemanni*, *E. xanthinum*, and *E. cochleatum*

(Goebel, 1920, p. 269), the flowers are non-resupinate. *Polystachya* and *Dendrochilum* are also genera in which the flowers of all the species examined are non-resupinate; *Polystachya* further appears to be peculiar since the flowers fail to twist round when the erect inflorescences are bent down. In other orchids examined, inversion of the flowering spikes or stems quickly results in a twisting of the flower to get the labellum into its normal position. *Osmoglossum pulchellum* with its erect spikes is somewhat peculiar, since the pedicels of the non-resupinate flowers are bent downwards sharply at right angles about half-way along their length.

As Goebel (1920, pp. 268–84) has dealt so fully with the resupinate and non-resupinate orchids, in some cases working with the same species with which unwittingly I had also been working, only one or two examples, to which he does not refer in detail, need be mentioned.

Cycnoches aureum (Ames, 1938, p. 159) has pendent racemes, but the flowers are non-resupinate, the labellum being in the superior position. The flowers, however, since the raceme is pendent, have had to twist through an angle of 180° in order to get the labellum uppermost—a comparable case to the pendent racemes of *Wistaria*.

In order to study the response of the flowers to a change in position of the inflorescence, a raceme in large bud was tied upright and in three days the flowers had twisted round to bring the labellum to the adaxial position. Some

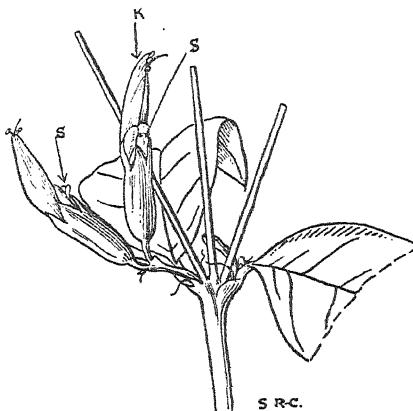


FIG. 3. *Brachysema lanceolatum* Meissn., showing the erect flowers with large keel (*k*) and small standard (*s*). (Nat. size.)

of the flowers twisted in the clockwise direction, thus completing the circle by making a complete twist of 360° , while others twisted counter-clockwise, thus untwisting the original torsion of 180° . The raceme was then released and allowed to hang down in its normal pendent position. Several of the flowers quickly responded to the change, especially those at the basal part of the raceme; the flowers were marked in order to observe the direction of the twist. In two cases the turning was clockwise and in one counter-clockwise. It would appear, therefore, that in this third twisting some flowers have undergone a further twist of 180° , making 540° in all (three twists of 180°), or, in the case of any flower which had untwisted when the spike was vertical, it has again made a twist through 180° . This agrees closely with *Gongora* (Bateman, 1865a), which Goebel (1920, p. 277) describes and figures.

Catasetum viridiflavum Hook. bears erect racemes with non-resupinate flowers, but these do not appear to have the power to respond when the inflorescence is inverted. The genus *Catasetum*, however, is a remarkable one in connexion with the phenomenon of resuspination, since certain species, such as *C. scurra*, have resupinate flowers on pendent racemes. The flowers, therefore, must have either twisted through 360° or untwisted the original 180° twist, while other species, *C. tridentatum* (Darwin, 1890, p. 193), bear non-resupinate flowers on erect stems.

C. barbatum Lindley, as Oakes Ames (1938, p. 161) points out, is particularly remarkable, since the female flowers are non-resupinate, but the male flowers, borne on the same inflorescence, are resupinate.¹

The cultivated varieties of the genus *Calanthe* are also of interest, not only for studying the complicated torsions of the individual flowers depending on their position on the raceme, which is erect below, then partly horizontal, and pendulous at the apex, but also on account of the rapid elongation of the pedicel of the individual flowers and its double curvature which takes place just as the flowers are opening. The pedicels increase from 1 cm. in length in the large bud stage to about 4·4 cm. when the flower opens in the course of a few days. These curvatures and growths can be well studied if an

¹ *Catasetum*. Subgenus *Clowesia*—flowers hermaphrodite, resupinate.

C. Russellianum and *C. Warscewitzii* (*C. scurra*) have pendulous inflorescences. In *C. roseum* and *C. dilectum* they are erect below and nodding at the apex.

Subgenus *Orthocatasetum*—flowers unisexual, rarely intermediate in characters, sometimes ♂ and ♀ on same inflorescence.

(a) Male and female flowers both non-resupinate.

Spike erect—*C. discolor*, *C. integerrimum*, *C. maculatum* (rarely the spike is pendulous), *C. purum*, *C. macrocarpum* (Ames, 1938, p. 166), *C. gnomus*.

Spike pendulous—*C. longifolium*; since the spike is pendulous the flowers have twisted through 180° .

(b) Male flowers resupinate, female flowers non-resupinate.

In these species the flowers must either have untwisted or performed a further twist of 180° (360° in all) depending on whether the spike is erect or pendent.

Spike ascending, nodding or pendulous—*C. pileatum* (*C. Bungerothii*), *C. laminatum*, *C. fimbriatum*, *C. barbatum* (Ames, 1938, p. 162).

Spike erect—*C. saccatum* (*C. Christyanum*) (or nodding in *C. Christyanum*), *C. planiceps*, *C. trulla*, *C. callosum*.

inflorescence is tied upright, when all the young buds will be seen to be 'upside down' when compared with the open flowers.

Perhaps the most remarkable orchid in connexion with flower torsion is *Angraecum eburneum* (Goebel, 1920, p. 278), whose flowers, like those of *Malaxis paludosa*, open in the non-resupinate position but in reality are doubly resupinate, having twisted completely round in order to get the labellum to its primitive position as the superior petal, the position which it occupies in the young bud stage. *A. eburneum* has a distichous inflorescence bearing some 12–14 flowers arranged alternately on the erect flower stalk. The flower buds in the young state are covered by their bracts and lie parallel to the main axis, with the labellum and nectary adaxial. As they grow and emerge from the protecting bract the pedicel elongates and turns downwards until it lies at right angles to the main axis. The flower bud also assumes the horizontal position, and as it does so the pedicel and ovary twist through an angle of 180° , so that the lip and nectary which were on the upper side of the flower are then on the lower side, and the bud is resupinate (Fig. 4, A). The torsion of the ovary and pedicel continues, but in a plane at right angles to the resupination twist, and this results in an inversion of the bud in the vertical plane bringing the bud again parallel to the main axis, but with the apex of the flower pointing downwards and the nectary pointing vertically upwards (Fig. 4, A–C); the lip and nectary being adaxial.

The buds on either side of the main stalk next turn towards the centre, those on the left-hand side of the stalk turning clockwise; while those on the right-hand side turn in the counter-clockwise direction (Fig. 4, C), the buds still remaining parallel to the main stem with the nectaries pointing upwards. This third twist being in a plane at right angles to the former one.

The buds then undergo a complicated twist in a plane again at right angles to the previous torsion and turn over from above (i.e. the nectary tip) downwards, in such a way that in the course of the twisting the nectary first becomes horizontal—pointing directly away from the flower stalk—and then, as the twist continues, the flower is carried *under* the ovary so that the nectary is brought to point downwards at an angle of 45° with the horizontal (Fig. 4, C–F). Finally, as the flower opens, the twist is completed and the nectary is directed vertically downwards and lies parallel once more to the main axis, but in the reverse direction (Fig. 4, F).

These singular torsions, or rather contortions, represent four distinct movements in four different planes. First the bud moves through an angle of 90° from the vertical to the horizontal position, secondly in conjunction with this movement it becomes resupinate, twisting through 180° , and the first movement is continued through a further 90° which inverts the flower in a plane at right angles to the resupination twist. There is then a twist of the flowers through 90° towards the main axis, followed by the final and second complete inversion of 180° in the vertical plane, making torsions of 630° in all.

The flowers of *Angraecum giryamae* and *A. superbum* exhibit exactly

similar torsions. Those of *A. sesquipedale* are resupinate as is well known, but it is of interest to record that on the large plant at Kew two spikes were borne this year (January 1939) on each of which one of the flower buds when well developed was still 'upside down', that is, with the labellum and coiled-

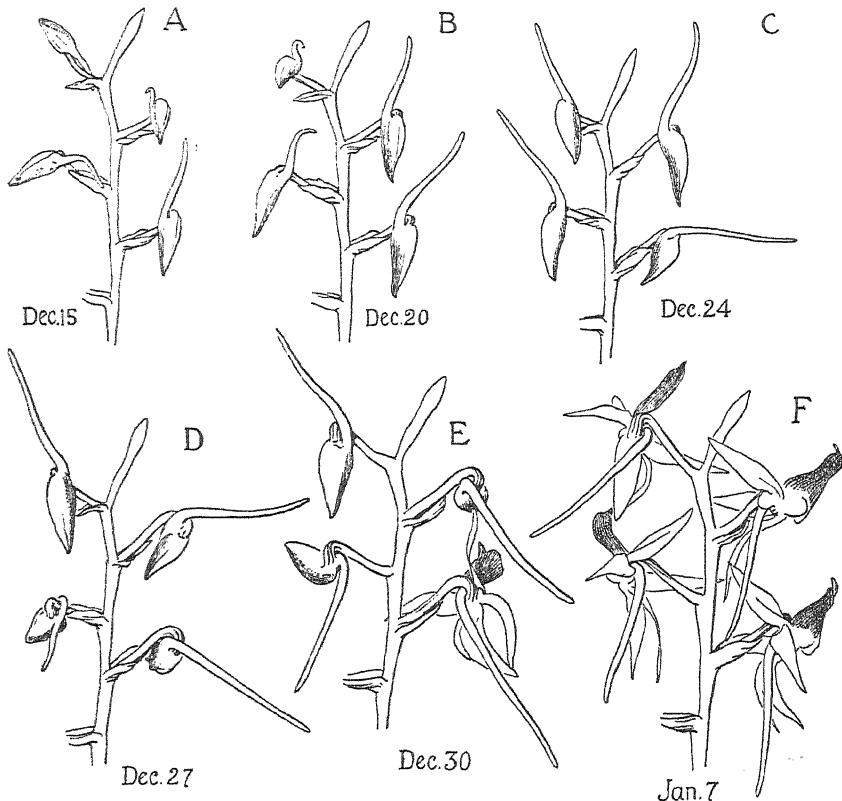


FIG 4. *Angraecum eburneum* Thou. Upper part of a raceme to show the various torsions of the buds as they develop. The drawings have been made from photographs taken by Mr. S. F. Ormsby at intervals from Dec. 15, 1938, to Jan. 7, 1939. The labellum or labellum side of the bud is shaded in every case.

up nectary on the upper side. In one case the ovary twisted in the clockwise direction and in the other counter-clockwise to bring about the inversion of the flower.

The torsions performed by the flowers of *Mormodes* are certainly as remarkable as any of those exhibited by orchid flowers. The flower has been well described and figured by Darwin (1890, p. 208), but nevertheless it seems worth while to describe the complicated twists in some detail. The resupinate flowers are born on an erect spike and thus make the usual twist of 180° in order to invert the position of the labellum. They then turn towards

the main axis, those on the left of the spike turning to the right and those on the right turning inwards to the left, through an angle of about 45° in the lateral plane. Further torsions then take place in the labellum and in the column. The labellum, as it lies expanded in the bud, is broadened above

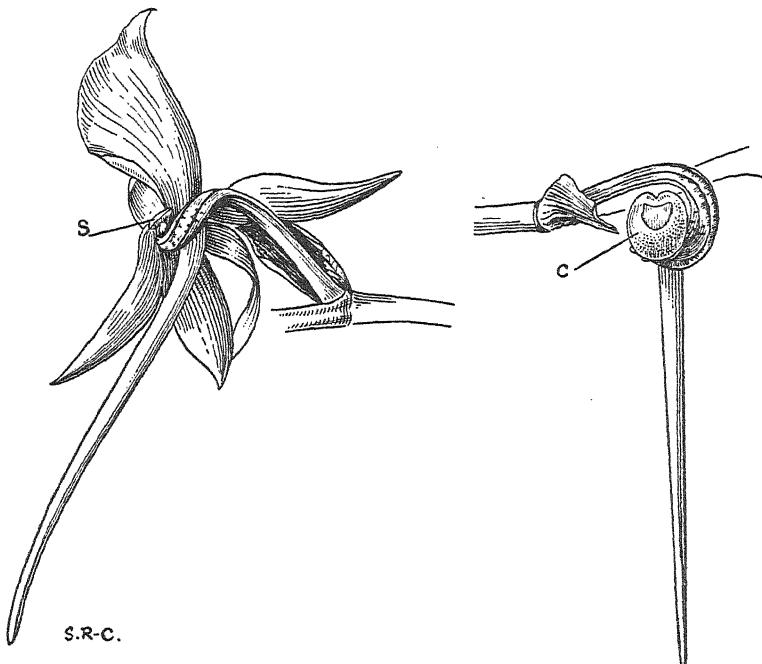


FIG. 5. *Angraecum eburneum* Thou. Open flowers to show the torsion of the ovary. *s.*, sepal removed; *c.*, column. The perianth has been removed from the flower on the right.

and narrowed into a foot-stalk at the base; the broader upper part is slightly concave owing to its width and the confining limits of the bud. When the bud opens the sides of the upper part of the labellum are reflexed and it becomes erected from the base, while the upper part bends over more or less at right angles to the foot-stalk and arches over the column.

The upper part of the labellum with its upturned edges thus resembles an inverted saddle, poised on a stalk shielding like a hood or roof the column below. The now inverted upper part of the labellum also bends sideways, the sideways twist being either to the left or to the right, depending on the position of the flower on the main stalk. The column also is curiously bent, the upper part being at right angles to the lower portion which lies horizontal and at right angles to the ovary; the upper part then twists about 90° laterally either to the left or to the right, depending on the position of the flower on the spike and on the position of the labellum above, so that the pollinia when expelled will not come in contact with the foot-stalk of the labellum. Thus

on looking at a spike of flowers of *Mormodes tigrina* Rodrig., which recently flowered at Kew (Fig. 6), the back of the column can be seen in the flowers on one side of the main axis, whilst those on the other side show the front of the twisted column.



FIG. 6. *Mormodes tigrina* Rodrig., showing the twisting of the column and lip in the flowers on either side of the main axis of the spike. Those on the left-hand side are twisted in the opposite direction to those on the right.

Finally attention may be drawn to the genus *Dendrobium* (Bateman, 1865 b) whose resupinate flowers accommodate themselves so beautifully to the various angles the stems may assume when attached to tree-trunks. The buds, as in all orchids, have the labellum adaxial and twist through 180° or more to orientate the flower so that the labellum is in the correct position for the visiting insect. In two Australian species of *Dendrobium*, however, *D. fusiforme* and *D. aemulum*, the buds on the erect racemes do not make a complete twist, with the result that the open flowers are more or less horizontal or sideways, since they have undergone a twist of only 90°. In this peculiar position the labellum does not seem to be in at all a satisfactory position as a landing-place for insect visitors.

Leaf Torsions

Leaf torsions are beautifully shown in the well-known cases of the genera *Alstroemeria* and *Bomarea* (Amaryllidaceae) (Arber, 1925, p. 68; Goebel, 1920, pp. 119-218). Less well-known examples are afforded by *Luzuriaga*

radicans (Ruiz and Pavon, 1802; Hooker, 1879), from Chile (Philesiaceae), *Leptaspis cochleata* and the other species of this genus, and by *Pharus latifolius* (Gramineae) and also by *Stylium pilosum* (Styliaceae) from Australia (Lindley, 1842).

In all cases the petiole has twisted through an angle of 180° so that the actual upper surface of the leaf is, morphologically, the lower surface. The twist not only reverses the leaf surfaces, but it also reverses the position of the constituents of the vascular bundles, so that in all these inverted leaves the phloem now faces the upper side of the leaf, whereas in a normal leaf it is adjacent to the lower surface. Figures of the leaf anatomy of *Pharus* are given by Goebel (1920, pp. 206, 207), who also gives a general account of the stomatal arrangements in *Alstroemeria* and *Bomarea*. *Leptaspis* shows stomata on both surfaces of its lamina, and the palisade tissue has also developed on the morphologically lower surface, in response, no doubt, to the inverted position of the leaf. *Luzuriaga*, which has leaves bright-green above and glaucous beneath, agrees with the previous examples with regard to the palisade tissue, which, however, is not very clearly differentiated, but the stomata occur on the morphologically upper surface (i.e. below), where they are to be found in pits between the epidermal cells.

It is a matter for speculation whether the leaves of *Luzuriaga* have twisted round to prevent undue evaporation because the stomata were on the upper surface or whether the stomata have been developed on what is now the lower surface and have been lost on what has now become the upper surface.

Bomarea Carderi Mast. and *B. patacocensis* Herb. afford excellent opportunities of studying the leaf torsions since the long climbing stems may be found either horizontal, hanging down, or climbing vertically upwards, and the leaves may be induced, at will, in one or two days to untwist or to make a double corkscrew-like twist. In whatever position a shoot may be fixed the leaves very quickly respond and very frequently they will perform a double twist (Fig. 7) in order to maintain the morphologically lower surface more or less at right angles to the incidence of light.

In conclusion the twisting of the pitchers of many species of *Nepenthes* is a matter of considerable interest. The pitchers of *Nepenthes* have been frequently described and excellently illustrated in many botanical works but, with the exception of Danser's account (1928) of the family, no attention appears to have been drawn to the fact that most, if not all, of the species of *Nepenthes* bear two kinds of pitchers, one of which is always twisted round through an angle of 180° owing to the twisting of the petiole or tendril between the pitcher and the flattened lamina-like phyllode basal portion of the petiole. Most pitcher plants in the earlier stages of their life-history bear their pitchers close to the ground or on short shoots, and the plant at this stage of its life-history is in the form of a rosette. The pitchers borne by such basal leaves are flask-shaped, swollen at the base, and narrowing towards the aperture. The 'lid' of the pitcher always faces the petiole or tendril, while the

petiole is so placed that it lies between or slightly to one side of the two flanges or ridges which run vertically up the side of the pitcher facing the petiole to which the pitcher is attached, as Danser describes. As the pitcher plant grows it develops strong, elongating shoots which scramble or climb among the shrubs and stems round about the plant. The pitcher plant is then

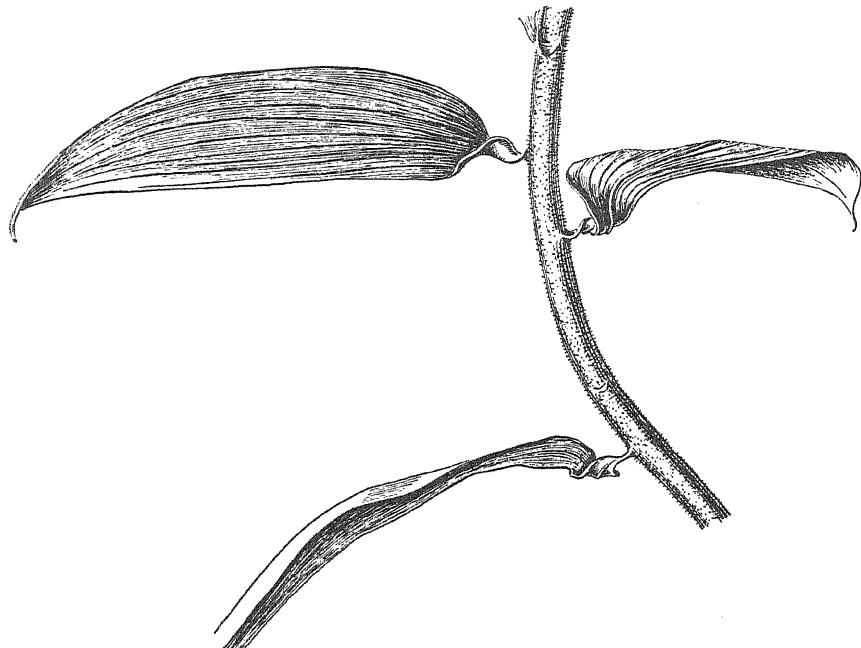


FIG. 7. *Bomarea Carderi* Mast. The shoot was at first pendent and then inverted and the leaf petioles have twisted twice in order to keep the morphologically lower surface of the leaf uppermost. Reduced.

in the climbing state or condition and later produces its flower spikes on these climbing shoots. When the plant has assumed the climbing condition which, as will be shown later, appears to be a definite physiological state, it naturally needs support and the petioles below the pitchers serve as tendrils and twist round whether there be any support for them to cling to or not. This twist of the petiole naturally reacts on the pitcher and causes it to be turned round through 180° , so that the petiole now lies against the side of the pitcher opposite the flanges and the 'lid' of the pitcher has its back to the petiole (Fig. 8). Both the lid and the orifice of the 'twisted-round' pitcher thus face away from the petiole instead of towards it, as they do when the plants are in the rosette or vegetative condition. Moreover, it will be noticed that the 'twisted-round' pitchers are funnel-shaped and taper somewhat towards the stouter base in contrast to the more swollen flask-shaped bases of the pitchers borne on the basal leaves of the same plant.

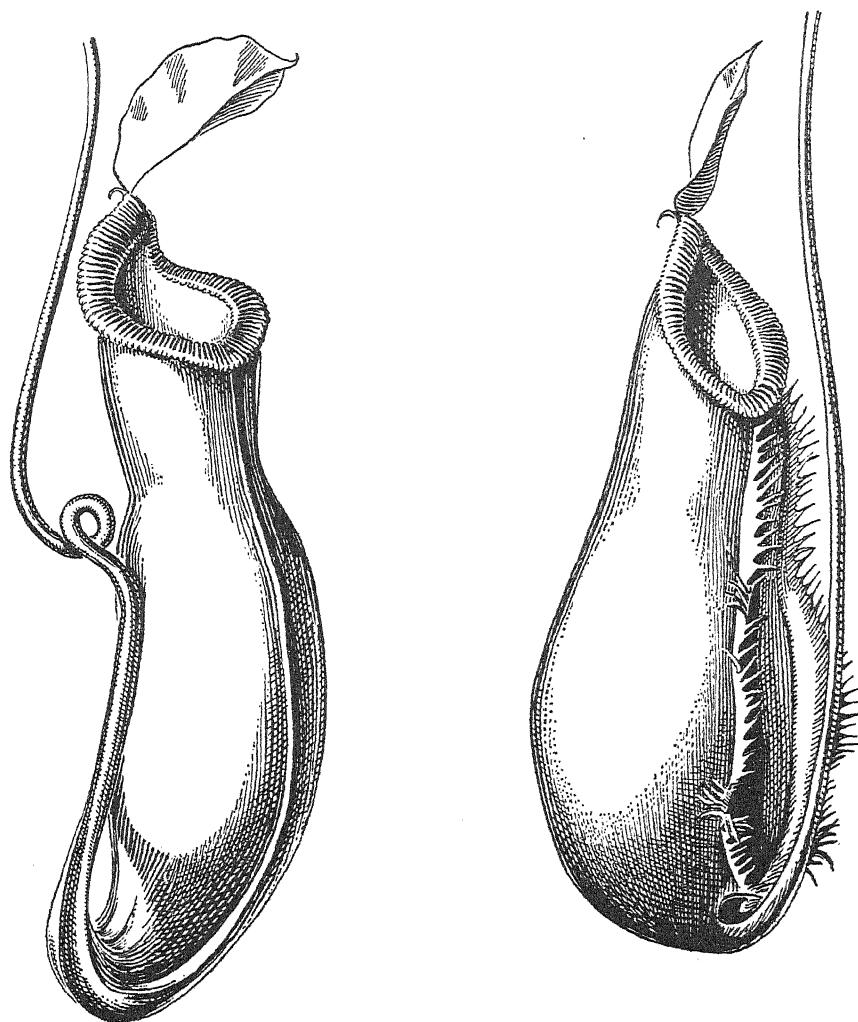


FIG. 8. *Nepenthes Henryana* Hort. (*N. Hookeriana* × *N. Sedenii*.) Two pitchers from the same plant; the one on the right from the base of the plant with the stalk of the pitcher lying between the flanges and the 'lid' and orifice of the pitcher facing the stalk. The pitcher on the left was borne on the upper climbing portion of the plant and shows the twist in the stalk which reverses the position of the pitcher. The flanges are now on the opposite side of the pitcher to the stalk and the back of the 'lid' is next to the stalk. The pitcher is also narrowed towards the base and not swollen at the base as is the case in the pitcher in the vegetative condition. Reduced.

Dr. B. H. Danser (1928) in his comprehensive account of the Nepenthaceae of the Netherlands Indies is the only writer on the family who appears to have drawn attention to the different forms of the pitchers and the torsions they exhibit as they grow wild. He describes three forms of pitcher, 'rosette pitchers' and 'pitchers of the short shoots', both of which are urceolate in shape, broader at the base, and with prominent flanges or wings which face the centre of the rosette, the stalk of the pitcher lying between the wings or slightly to one side of them in the case of the 'short shoot pitchers'; the lid of the pitcher being always attached to the opposite side of the pitcher to the two flanges or wings. The back of the lid in these pitchers, as already mentioned, thus faces outwards. His third type is the 'pitcher of the climbing stems', which are more tubulose or infundibuliform with the wings reduced to prominent ribs, as is the case with plants under cultivation, the pitchers being twisted round owing to the torsion of the 'petiole' or 'tendril'. Excellent figures are given by Danser of the pitchers borne by the climbing stems of many of the Dutch East Indian species.¹

When a pitcher plant has developed the climbing habit it will continue to produce leaves with twisted petioles or tendrils, the pitchers, of course, when developed, being twisted, though many of these leaves may not produce pitchers at all. The plant in fact has passed over into a particular physiological condition which may be referred to as the adult or flowering state—a state which has been fixed—since it appears, when it has passed into this state, to be unable to revert to what may be termed the vegetative condition in which the pitchers are untwisted. That this is the case is shown by the fact that cuttings taken from plants under cultivation, when they have reached the climbing or adult state, will always produce reversed pitchers and never pitchers like those borne by the basal leaves of a plant in the vegetative condition. In order, therefore, to reproduce a normal pitcher plant it is essential to take cuttings from that part of a plant which bears untwisted pitchers.

A further indication that the plant, when it has developed its climbing shoots, is in a fixed physiological condition is afforded by the fact mentioned above that the tendrils or petioles of the leaves, whether they terminate in pitchers or not, always exhibit a spiral twist though there may be no support near at hand.

Comparable cases of plants which pass into an adult state or fixed condition which appears to be connected with flowering and reproduction are worthy

¹ See Fig. 1, p. 278, *N. carunculata* Dans.; fig. 2, *N. clipeata* Dans.; fig. 3, *N. decurrens* Macf., and many others.

Figures of the short shoot pitchers are also given for *N. Hookeriana* Lindl. (Fig. 9, c, p. 310), *N. leptochila* Dans. (Fig. 13, c, p. 320), *N. papuana* Dans. (Fig. 16, c, p. 348), and *N. pectinata* Dans. (Fig. 17, d, p. 350).

Good pictures of both kinds of pitchers will be found in the various works cited in the literature (Beccari, 1896; Hemsley, 1906; Le Maout and Decaisne, 1873, pp. 703, 704; Lindley, 1846, p. 287; McFarlane, 1908; Masters, 1880; Nicholson, 1888, ii. 435; Veitch, 1898), but in no case is any explanation offered as to the reason for some of the pitchers being reversed.

of record in connexion with the vegetative and climbing states of *Nepenthes*; one of these is the common Ivy, *Hedera helix*. Ivy climbing on a wall or on a tree, if left undisturbed, will in course of time put out short shoots which grow away from the support in the form of short, stout shoots with a rosette arrangement of the leaves on the stems. These short, bushy shoots bear the umbels of flowers, and if cuttings be taken from such flowering shoots the resultant plant will form bushes only and will not produce any climbing shoots. In this way, as gardeners well know, the 'Tree Ivies' or 'Bush Ivies' have been produced and such bushes will constantly bear short flowering shoots and remain in the fixed physiological condition of the flowering branches from which they have been taken.

Ficus repens behaves in a precisely similar manner. Cuttings taken from the strong, free-growing, bushy shoots, which are formed if the climbing specimens are allowed to grow freely, always result in bushy, non-climbing plants.

A further case is afforded by *Vitis gongylodes*, which towards the end of its actively growing vegetative period produces flowering shoots. Cuttings taken from such flowering shoots will produce only flowering plants, so that if cuttings have not been made from the vegetative shoots one cannot regain the climbing plant and the species, should there be only one plant in cultivation, would be lost.

The torsions characteristic of orchids and other flowers which have been described, and those exhibited by leaves, though they are in both cases reversions of the normal positions both of the flowers and leaves, have no doubt come about as a result of quite different stimuli.

In the case of the flower torsions they can best be explained in connexion with the visiting insects which pollinate them, though the reason of the complete inversion of the flowers in such genera as *Malaxis* and *Angraecum*, which result in the flower regaining the position it would have occupied had no twisting occurred, is difficult to explain. The only possible suggestion would seem to be that having, like the majority of orchids, the instinct to twist the flowers into the resupinate position, some further adaptation to the proper visiting insect gradually developed and, instead of untwisting to meet the demand, the flowers, owing to some physiological condition or impulse, continued the twisting and so carried the labellum round—boxing the compass—until the full circle had been accomplished by a twist of 360° .

Some physiological stimulus must also be sought to account for the leaf torsions referred to and the pitcher twisting in *Nepenthes*. In the case of the leaves, light or transpiration may have been the controlling factors, but in the case of *Nepenthes* the torsion of the pitchers appears to be associated with a definite physiological state into which the plant passes when it becomes adult and develops its climbing stems which ultimately bear the reproductive organs.

SUMMARY

The torsion performed by zygomorphic flowers borne on pendulous racemes—such as Wistaria, Laburnum, &c.—is described and examples are given of the torsions which such zygomorphic flowers will exhibit if normally erect spikes are turned vertically downwards or normally pendent ones tied erect.

An account is given of orchid flowers which normally are non-resupinate—species of *Epidendrum*, &c.—and of those which are resupinate and of the further torsions which are involved when inflorescences bearing resupinate flowers are pendent. The torsions exhibited are illustrated by reference to the pendulous racemes of Cycnoches and to the flowers of *Mormodes tigrina*. The complete inversion of the flowers of Malaxis, which thus become ‘non-resupinate’, is mentioned and a full account of the similar inversion of the flowers of *Angraecum eburneum* is given, where the complicated torsions in three different planes result in a torsion through 630°.

The leaf torsions of Bomarea, Luzuriaga, Pharus, and Leptaspis, where the morphologically lower side of the leaf becomes the upper surface owing to the twisting of the petiole, is described and an account is given of the two different types of pitcher in Nepenthes—those of the climbing stems being reversed owing to the twisting of their petioles. The physiological state into which the pitcher plant appears to pass when it reaches the adult condition, is compared with the production of Tree Ivy, which is propagated from the short, flowering shoots of the climbing plant.

My thanks are due to Dr. C. R. Metcalfe for his assistance in examining the anatomy of the leaves of Leptaspis and Luzuriaga; to Mr. L. Stenning and Mr. W. E. Everett for their help in connexion with the orchid studies; to Miss Ross-Craig and Mr. G. Atkinson for their drawings, and to Mr. S. F. Ormsby for taking numerous photographs to show the twisting of the flower buds of *Angraecum eburneum* and other plants which have been reproduced as drawings for the text figures. Mr. V. S. Summerhayes kindly furnished me with the particulars relating to the different species of Catasetum and other orchids.

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Further Studies on Transport in the Cotton Plant

VII. Simultaneous Changes in the Production and Distribution of Dry Matter under Varying Potassium Supply¹

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With five Figures in the Text

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I. INTRODUCTION

IT seems established that potassium plays a rather special role in promoting carbon assimilation (cf. Rohde, 1936; Pirsche, 1938). There is also some evidence that transport is hastened by this element (cf. Maiwald and Frank, 1935). How potassium affects these processes is not known. There is also a dearth of information concerning the quantitative relations of these processes at different levels of potassium uptake by the plant. What information there is appears to be limited to the effects of potassium starvation. The work of Gregory and Baptiste (1936) suggests that translocation and carbon assimilation may be affected to the same extent by a deficiency of potassium. How these processes are affected by an excess of potassium is not apparently known.

In the course of some work on the partition of the mineral elements (Phillis and Mason; in the press) a remarkable relationship between the production and distribution of dry matter over a wide range of potassium supply came to light. While the dry weight of the plant may be affected by factors

¹ Paper No. 20 from the Physiological Department of the Cotton Research Station, Trinidad.

other than carbon assimilation, and while the distribution of dry matter between the leaf lamina and the rest of the plant may be affected by factors other than transport, the relationship discovered between the production and distribution of dry matter is so striking that a clue, it is believed, is offered to the way in which potassium affects photosynthesis and transport.

II. EXPERIMENTS IN SAND-CULTURE

A. Procedure.

Four experiments are described in this section, two with varying potassium supply and one each with varying nitrogen and phosphorus. The plants were grown in sand-cultures with a full nutrient solution in two glass-houses, which will be referred to as houses A and B respectively. The results for each house are recorded separately. Approximately 150 plants were grown in each house. The plants were 9 weeks old at the time of sampling and had just begun to flower.

To express the distribution of dry material between the leaf lamina and the rest of the plant we have followed the procedure of Gregory and Baptiste and have used the ratio Rest of Plant/Leaf Lamina. Roots are of course included with the 'Rest of Plant'. This ratio we shall refer to as the *Distribution Index*. The results for Dry Weight, *Distribution Index*, and Uptake of the Mineral Elements (potassium and nitrogen only) for the Whole Plant are shown graphically (Figs. 1 and 2) as percentages of the mean values for the whole range of potassium, nitrogen, or phosphorus supply as the case may be.

B. Results.

The results for the two potassium experiments are shown in Fig. 1. The potassium supply to the roots varied in both experiments from 12.5 p.p.m. to 400 p.p.m. Results for the uptake of potassium by the whole plant are available for the first experiment only, and are reproduced with the dry weights and the *distribution indices* for this experiment on the left of Fig. 1. It will be seen that the potassium uptake by the whole plant showed a much greater range than either the dry weight or the *distribution index*. In both experiments and in both houses the changes in the *distribution index* are almost the same as those for the dry weight. Moreover, there is a remarkable correspondence in the pattern of the curves for the dry weights and the *distribution indices*.

It is important to notice that the two curves follow one another, not only under conditions of potassium starvation but also under conditions of potassium excess. It will be evident that whatever factors are controlling the production of dry matter are also controlling the distribution of dry matter, and that this applies from conditions of potassium starvation to conditions of excess. The correlation coefficients between dry weight and the *distribution index* are shown on the graphs. The correlations are positive and almost perfect and are fully significant ($P < 0.05$).

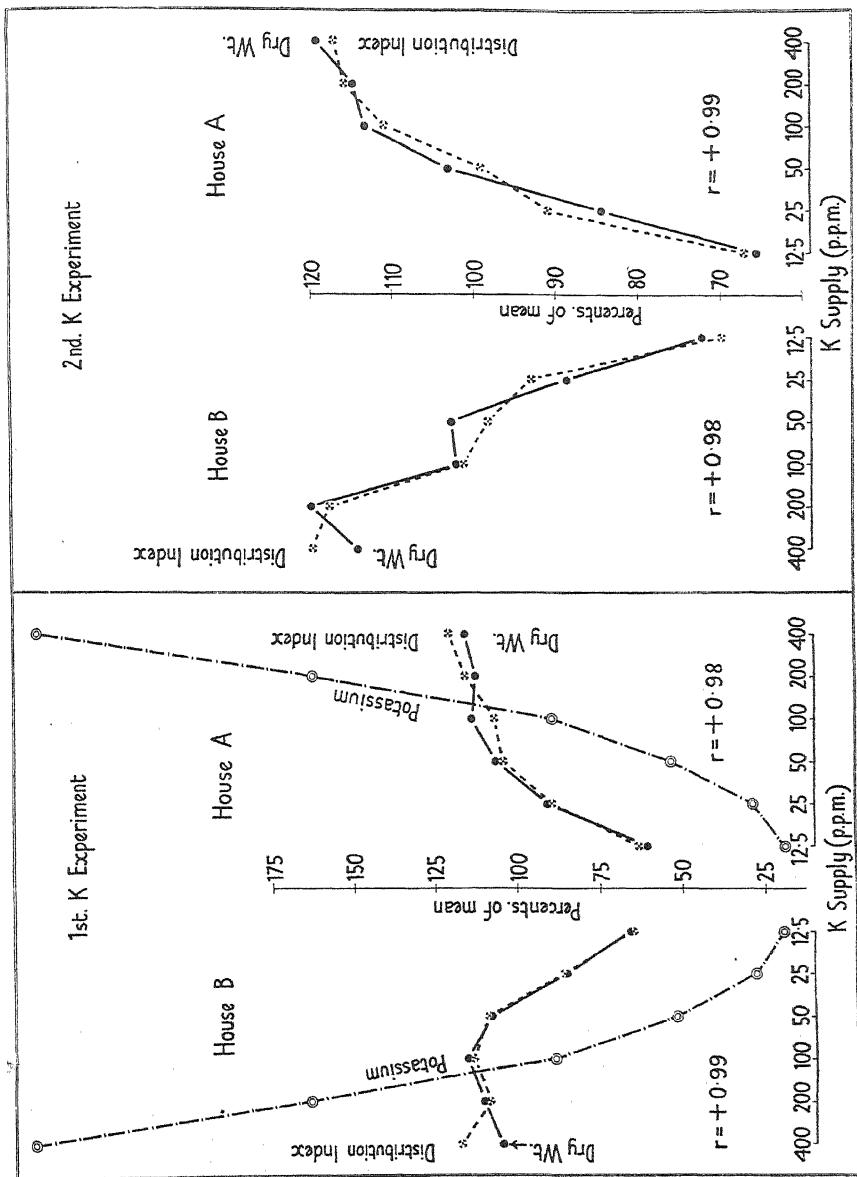


FIG. 1. Dry weights and distribution indices plotted against potassium supply, also correlation coefficients between dry weight and distribution indices, for 1st and 2nd potassium experiments. In the first experiment the uptake of potassium is also shown

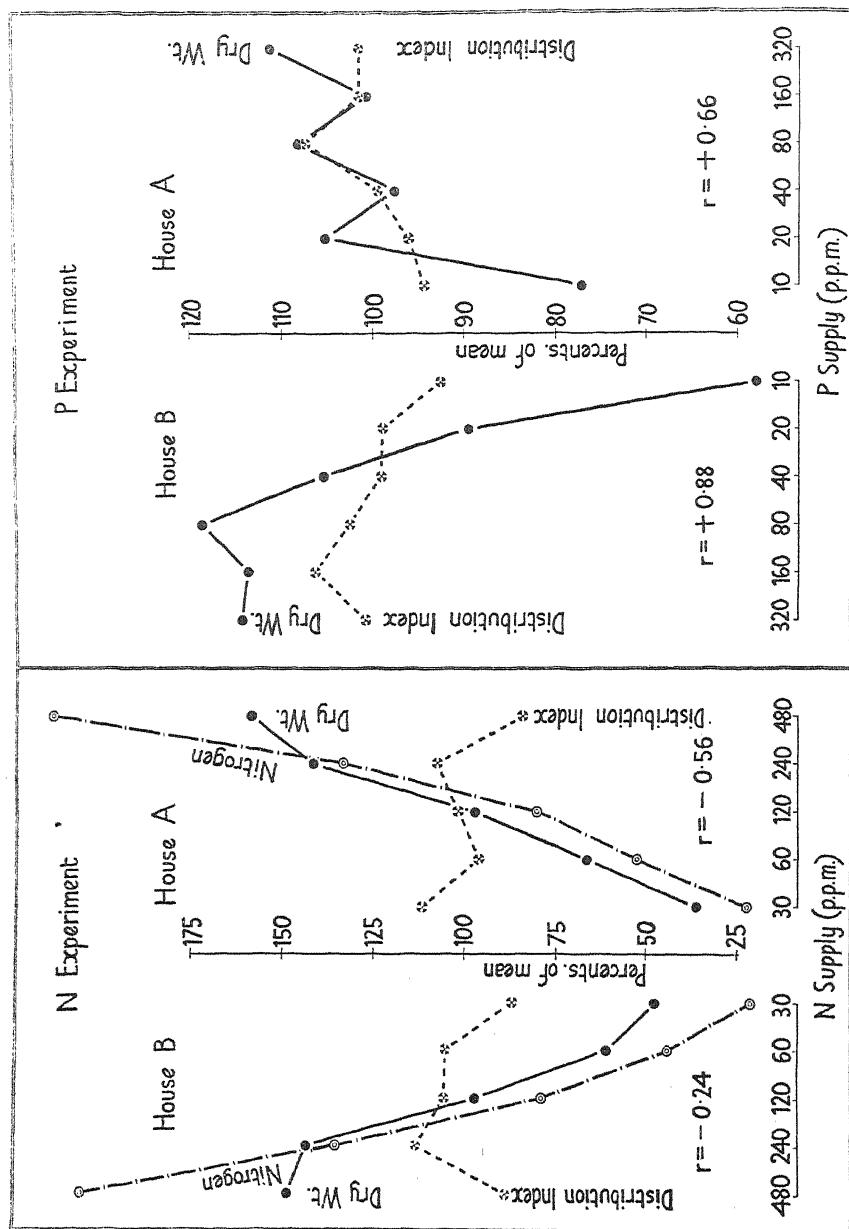


FIG. 2. Dry weights and distribution indices plotted against supply of nitrogen (left) and phosphorus (right); also correlation coefficients between dry weights and distribution indices. In the N experiment the uptake of this element is shown, between dry weights and distribution indices.

The changes in dry weight and *distribution index* under conditions of varying nitrogen and phosphorus supply respectively are shown in Fig. 2. For nitrogen the results are quite unlike those just reported for potassium. Inspection of the graph will make it clear that the two curves are quite unrelated. The correlation coefficients which are shown on the graph are of opposite sign and do not attain the level of partial significance ($P = 0.10$). The uptake of nitrogen by the whole plant is shown on the graph, but results are not available for the uptake of phosphorus.

Under varying phosphorus supply there is some suggestion that both dry weight and *distribution index* are affected in the same way. The correlation coefficients are both positive, that for house B is fully significant, while that for house A is not even partially significant. Gregory and Baptiste, however, report that phosphorus while promoting photosynthesis has the reverse effect on transport.

III. EXPERIMENTS IN THE FIELD

A. Procedure.

In these experiments the plants were grown on a light loam in the open. There were two experiments arranged side by side. In one, a basal dressing at the rate of 370 lb. of nitrogen and 196 lb. of phosphorus per acre was applied before the seed was sown. This experiment will be referred to as the N. P. Series. In the other there was no basal dressing. It will be referred to as the Normal Series. In each experiment there were five levels of potassium supply ranging from 0 to 40 cwt. of potassic fertilizers (mixed chloride and sulphate) per acre. The field was divided into five blocks, each of which was subdivided into ten plots of approximately $1/35$ th acre. Five of these in each block were used for the Normal and five for the N. P. Series. Each treatment was therefore replicated five times. The plants were again about 9–10 weeks old at the time of sampling.

B. Results.

The changes in dry weight and *distribution index* expressed as percentages of their mean values are shown in Fig. 3. It will be seen that both increased as a result of the application of 5 cwt. of the potassium fertilizer. The increase in the *distribution index* was, however, much less than that of the dry weight. It will be noticed that after the 5 cwt. level, heavier applications led to actual declines in the dry weight and *distribution index*. In the N. P. Series there is a distinct similarity in the pattern of the two curves. The correlation coefficients are not as high as in the sand-culture potassium experiments, but are nevertheless considerable and are both fully significant. The main interest of this experiment is that it demonstrates the existence of the correlation under conditions of excess potassium supply.

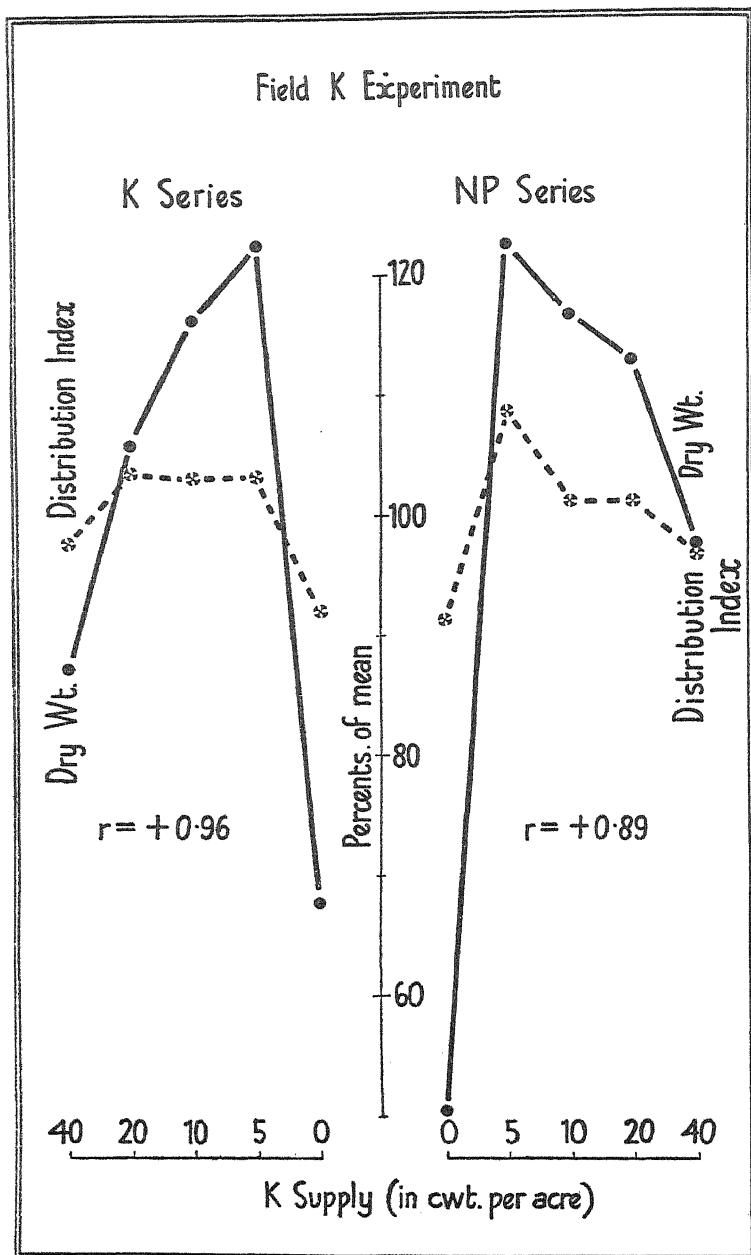


FIG. 3. Dry weights and *distribution indices* plotted against potassium supply, and correlation coefficients between dry weights and *distribution indices*, for potassium experiments in the field.

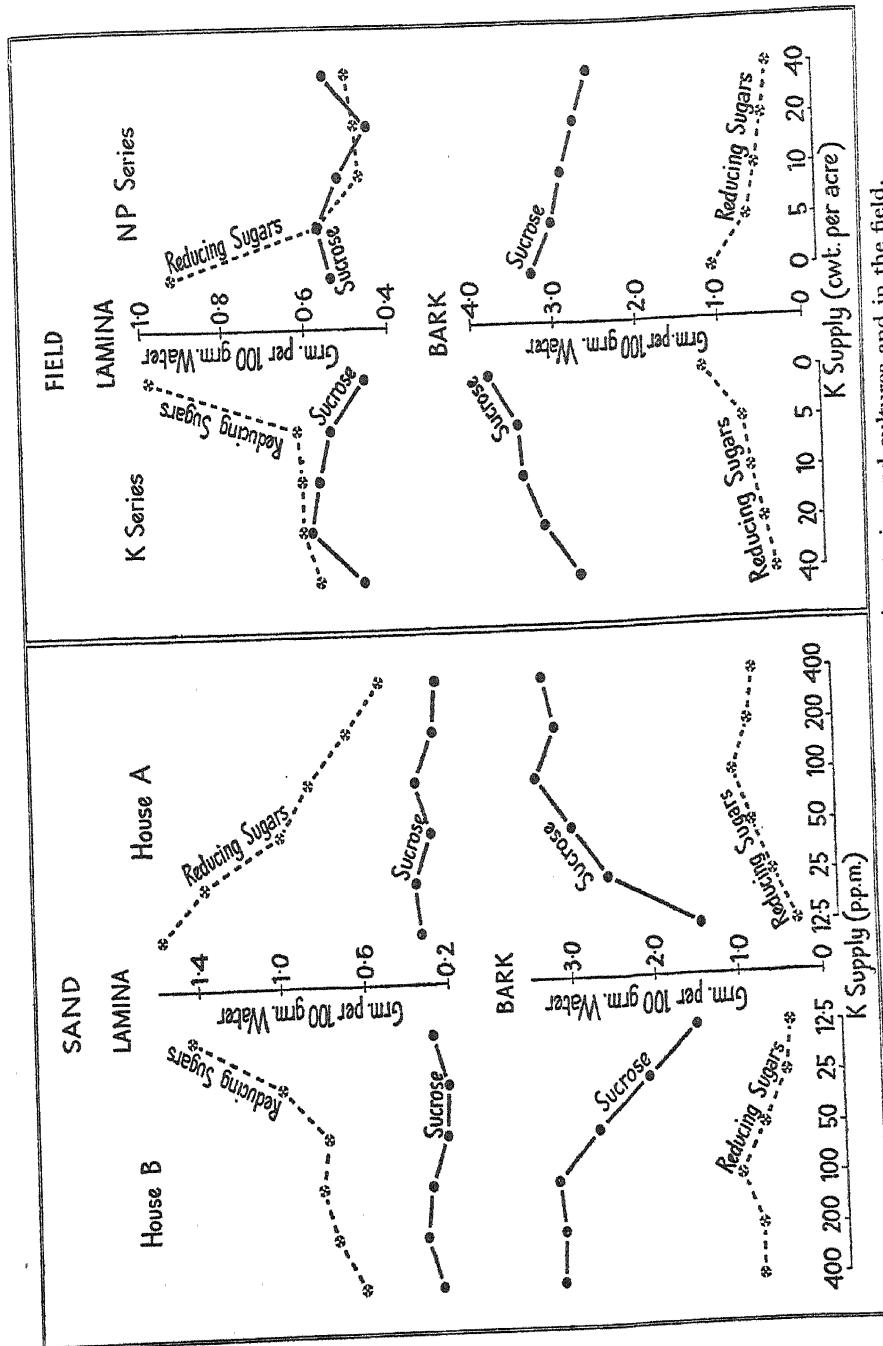


FIG. 4. Sugar concentrations in potassium experiments in sand cultures and in the field.

IV. SUGAR CONCENTRATIONS

The concentrations of sucrose and reducing sugars in the leaf lamina and in the bark for the first sand-culture potassium experiment (section I) are shown in Fig. 4 (left). The sugar determinations were as usual (Phillis and Mason, 1933) those of sap expressed from previously frozen tissue. The concentrations of sucrose were much greater in the bark than in the lamina. Potassium supply had no effect on the sucrose concentration in the lamina, but in the bark it markedly increased the concentration up to 100 p.p.m. supply. The concentration of reducing sugars in the bark was affected in much the same way as sucrose, but an increase in potassium supply was accompanied by a reduction in the concentration of reducing sugars in the lamina. It is interesting to note that at low levels of potassium supply the concentrations of reducing sugars in the lamina exceeded those in the bark. The results for the second sand-culture potassium experiment were essentially the same and are not presented.

The sugar concentrations in the lamina and in the bark for the field potassium experiments (section III) are also shown in Fig. 4 (right). The results for the lamina are much the same as those of the sand-culture experiments and do not call for special comment. The concentrations in the bark on the other hand behaved in a reverse manner to those of the sand-culture experiments; an increase in the supply of potassium to the roots was accompanied by a reduction in the concentration of sucrose and reducing sugars.

V. DISCUSSION

The salient fact disclosed by the experiments reported in this paper is the intimate relationship existing between changes in dry weight and in the *distribution index* over a wide range of potassium supply. The correlation holds both under conditions of potassium starvation and under conditions of excess, and in the sand-culture experiments is nearly perfect. This does not appear to be the case for nitrogen, where large changes in dry weight were not accompanied by any significant changes in the *distribution index*. There is some suggestion that both the production and distribution of dry materials are affected by phosphorus, but the correlations are much smaller than for potassium. The intimate relationship under varying potassium supply which is suggested by the correlation coefficients between dry weight and the *distribution index* is reinforced by the close agreement between the patterns of the curves for the production of dry matter and its distribution.

The quest for a unifying factor which under varying potassium supply would equally affect both the production and distribution of dry material is not an easy one. The search for such a factor is, however, narrowed somewhat by consideration of the behaviour of the leaf and of the bark in response to variations in potassium supply. In Fig. 5 are shown the changes in dry

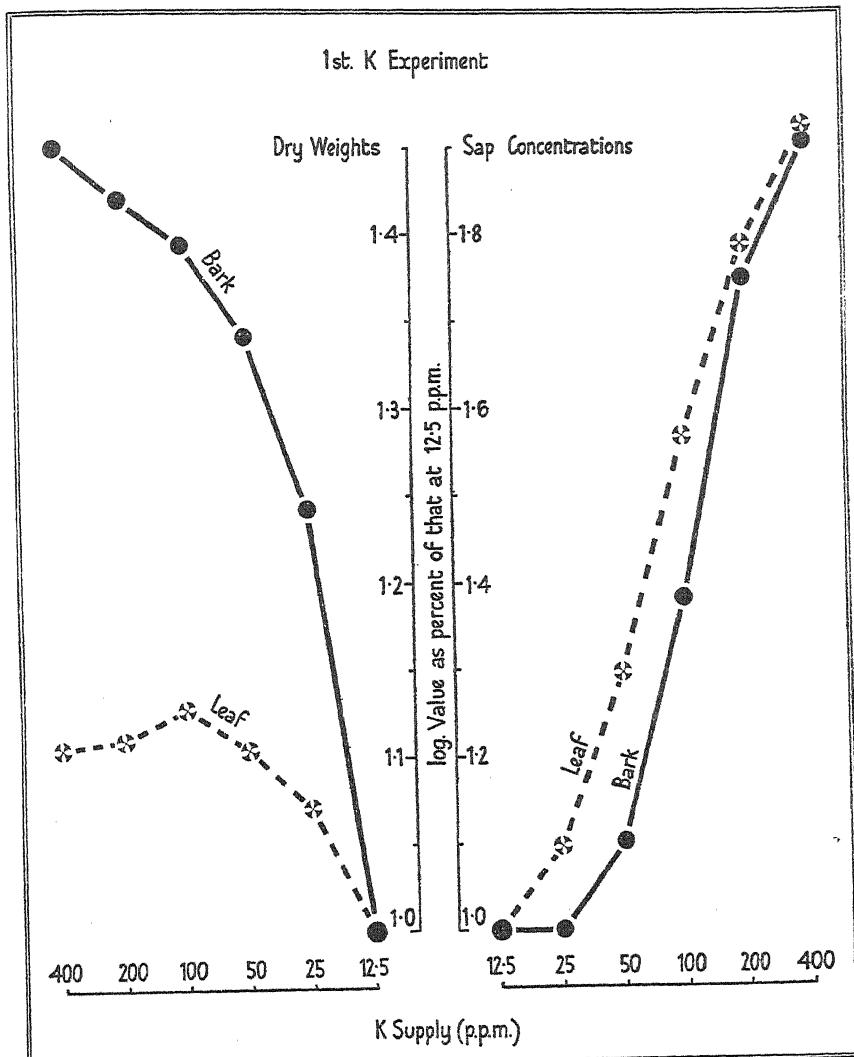


FIG. 5. Dry weights and potassium concentrations in sap of leaf and bark for 1st potassium experiment.

weight and in the concentrations of potassium in the sap of the leaf and of the bark of the first sand-culture potassium experiment (section I). The values represent the means for the two houses. It will be seen that the dry weight of the bark increased to a much greater extent than that of the leaf as the supply of potassium was increased. On the other hand, the concentrations of potassium in the leaf increased, at least initially, at a greater rate than those of the bark. If attention is focused on the changes between the 12.5

p.p.m. and the 25 p.p.m. levels of potassium supply, it will be seen that the bark increased its dry weight by 75 per cent. without the occurrence of any change in its concentration of potassium. There was actually, it may be added, a small loss in the weight of potassium per 100 gm. dry weight. Other non-foliar tissues behaved like the bark in showing no increase in the weight of potassium per 100 gm. dry weight between the 12·5 p.p.m. and the 25 p.p.m. levels. It would thus appear that the great increase in dry weight in the bark cannot be directly due to potassium. It would follow that the cause of this great increase in dry weight in the bark must be due to some factor in the leaf. As the phloem of leaf and bark are continuous and as the bark is largely composed of this tissue, it would appear that the phloem as a whole cannot be the cause of the changes in the *distribution index* under varying potassium supply. There remains the whole leaf parenchyma between the phloem of the veins.

If we are justified in excluding both the phloem and the bark as a whole, and in seeking for the cause of the changes in the distribution of dry matter under varying potassium supply in the leaf parenchyma, then a factor unifying both the changes in the production and distribution of dry materials presents itself in the diffusion constants (Mason and Phillis, 1937) for solutes of the protoplasm of the leaf parenchyma. This hypothesis assumes that the dry weight is affected by changes in the potassium supply as a result of changes in photosynthetic activity which are in turn due to the effect of potassium on the diffusion constants of carbon dioxide moving to the chloroplast and of sugars moving away from it. The change in the diffusion constant of sugars in the leaf parenchyma will also affect the distribution of sugars between the leaf and the rest of the plant. The rate at which carbohydrates move from the chloroplast to the phloem of the bundle ends will be affected to the same extent as the movement of carbon dioxide from the surface of the cell to the chloroplast.

It must be admitted that it is by no means easy to harmonize this hypothesis with the changes observed in the sugar concentrations of the leaf and of the bark (see Fig. 4). It is true that fuller sugar analyses, chemical, regional and throughout development, might make the task simpler. One fact that does, however, emerge from the examination of the sugar concentrations, is that potassium must alter either directly or indirectly not only the diffusion constants of carbon dioxide and sugar in the leaf but also the whole machinery responsible for the utilization of sugar in the plant. It would appear that changes in the index of sugar utilization (Maskell and Mason, 1930) of a tissue or of different tissues, though they must affect the concentration of sugar in the tissue, need not appreciably affect the production of carbohydrate by the plant nor its distribution between different tissues.

Before closing, a word should be said concerning the changes in dry weight that occurred under varying potassium supply. The dry weight increments at low levels of supply were relatively large and gradually diminished as the

supply was increased until, with excess, they became negative. This is true both of the whole plant and of the leaves. One explanation of this decline in the dry weight increments is that factors other than potassium assumed control of dry weight production. If this is the correct explanation, it would follow that they also assumed control of the distribution of dry matter and that they affected both equally. As this would involve the assumption that there are factors other than potassium that affect equally the production and distribution of dry matter, the possibility must be envisaged that the slackening off in dry weight production under increasing potassium supply was in some way due, not to other factors, but to an actual excess of potassium.

VI. SUMMARY

1. In this paper observations are recorded on the changes in the dry weight and in the distribution of the dry weight between the leaves and the rest of the plant under varying supplies of potassium, nitrogen, and phosphorus. It was found that dry weight and its distribution (rest of plant/leaf lamina) are highly correlated under varying potassium supply. This correlation holds both under conditions of low as well as under conditions of high potassium supply. No such correlation was observed for nitrogen, while the results for phosphorus were inconclusive.
2. This correlation was interpreted as being due to the existence of a quantitative relation under varying potassium supply between the rate of photosynthesis and the rate at which materials are exported from the assimilating cells to the phloem of the leaf.

3. It was suggested that potassium controls the rate of photosynthesis by altering the rate at which carbon dioxide diffuses to the chloroplasts and that it controls equally the rate of sugar export from the chloroplast to the phloem of the leaf by altering the rate at which sugar diffuses through the protoplasm of the parenchyma.

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Excretion of Nitrogenous Substances from Root Nodules: Observations on Various Leguminous Plants

BY

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AND

J. BOYES²

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I. INTRODUCTION

RECENT work has indicated that the nitrogen economy of a nodulated leguminous plant is not always of the same type. Of the nitrogen that is fixed in the nodules, sometimes the whole is retained within the plant and nodule system until the completion of the life-cycle. This has been shown to be the case for Soya bean plants growing in sand-culture under cool green-house conditions in west Scotland (Bond, 1938a), while the same conclusion has been reached for various leguminous species at other stations (see below).* When there is this complete retention of the fixed nitrogen, observations on Soya bean (Bond, 1936) show that the bulk of the fixed nitrogen is transferred with little delay from the nodules to the remainder of the plant.

From the work of Virtanen and his collaborators (see Virtanen, 1936a, b, and earlier papers) it is equally certain that in other circumstances the fixed nitrogen is not wholly retained by the leguminous plant; on the contrary a proportion, sometimes very considerable (e.g. 80 per cent.), leaks out or is excreted into the rooting medium. In view of its possible agricultural significance and of the opportunity which it provides for the further investigation of leguminous symbiosis in its general aspects, a number of attempts have been made to observe this phenomenon of excretion at other experimental stations. It appears that at few other stations has the same degree of success been obtained as at Professor Virtanen's laboratory at Helsinki: from

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some stations come negative reports only. At the Wisconsin station Wilson and his collaborators (Wilson and Burton, 1938) were at first unable to secure evidence of excretion, despite extensive trials. Latterly they have been able to detect excretion in several leguminous types (Wilson and Burton, loc. cit.¹; Wilson and Wyss, 1937), especially when the plants were exposed to special environmental conditions. At Rothamsted, Thornton and Nicol (1934) obtained evidence of a transfer of nitrogen from lucerne to rye grass; at the same station Nowotnówka (1937) secured evidence of uptake of nitrogen by barley plants from peas, though negative results were yielded with lucerne and clover. The same investigator had previously secured positive results with all three leguminous plants in experiments in Poland. Ludwig and Allison (1936) at Washington, in an extensive series of experiments involving nearly twenty different leguminous plants and cereals, obtained almost uniformly negative results. At the Waite Institute, South Australia, pot experiments by Trumble and Strong (1937) failed to reveal any very marked evidence of excretion of nitrogen during the vegetative growth of clover and lucerne, although previous observations in the field had suggested that excretion did occur there. Lastly the present authors have conducted a number of experiments in Glasgow. Some results, negative in nature, have already been published (Bond, 1938a); others are presented below.² Though they also are essentially negative no apology is required for their presentation, for only by a consideration of both positive and negative experiments can the conditions which promote excretion be determined.

II. METHODS

These experiments were of an exploratory type, designed to cover a wide range of plant material and conditions, and it is for this reason that in many cases the number of replicates was not all that could be desired. The experiments relate to varieties of the following leguminous plants: Soya bean (*Glycine Soja* Sieb and Zucc.), pea (*Pisum sativum* L.), and broad bean (*Vicia Faba* L.). Varieties of Soya bean included 'Manchu' (late flowering) and three which are now being grown in Great Britain to some extent, namely, 'Jap', 'Brown C', and 'Brooks', all early flowering in these latitudes. Varieties of pea included 'Gradus' (early maturing), 'Gladstone' (late), 'Maple' field pea (late), and two Finnish types, 'Torstag' and 'Concordia'. Bacillus strains for peas included HX (from Prof. Virtanen) and GW (isolated locally), and for Soya bean strains Nos. 9 and 505 from the Wisconsin collection. The strain used for Broad bean (BB) was isolated locally.

Soya beans were grown in a greenhouse which was unheated after the first week in May; during the period of the experiments the day temperature

¹ In this paper reference is also made to experiments with positive results carried out by Dr. Wilson at Helsinki.

² Preliminary statements regarding these results have already appeared (Bond, 1937, 1938b).

in experiments with peas (non-nodulated) growing in water culture, a slight loss of nitrogen from the plants to the culture solution.

In Expt. II (Table I) the fine quartz sand was used, and though the usual small increases in sand-nitrogen were observed, they were of much the same magnitude in inoculated and control pots, so that there is no evidence of

TABLE II
Associated-growth Experiments. Fine Quartz Sand

Pot No.	Bacillus strain.	Dry wt. of leguminous plants (gm. per pot).	N fixed by leguminous plants (mg.).	N content of associated plants (mg.).	Plants present.
Expt. III.*					
1	9	9.7	165	6.5	Manchu Soya bean and barley.
2	„	Similar to Pot No. 1.		6.3	Manchu Soya bean and barley.
3	„	5.4	84	6.5	Jap Soya bean and barley.
4	„	6.6	132	5.8	Brown C Soya bean and barley.
5	„	9.0	150	5.6	Brooks' Soya bean and barley.
6	<i>Control</i>	—	0	6.1	Manchu Soya bean and barley.
7	HX	4.5	160	7.0	Maple pea and barley.
8	„	2.3	90	12.0†	Gladstone pea and barley.
9	„	2.1	60	7.7	Torstag pea and barley.
10	„	4.3	120	7.3	Concordia pea and barley.
11	BB	—	350	No gain	2 Longpod Broad bean and barley.

* April 20-July 23, 1938. Glazed pots containing 3.2 kg. fine quartz sand. Three leguminous and 5 barley plants per pot. N content of 5 barley grains = 5.8 mg. Leguminous plants flowering or with pods at harvest, depending on variety. Ears of barley emerged.

† See text.

excretion from either peas or Soya beans. Growth and fixation were satisfactory in this experiment.

A few Soya beans and peas were grown with the root systems in closed containers, consisting of 2-litre flasks filled with the fine quartz sand and plugged with cotton-wool, an arrangement employed in many of Virtanen's experiments although in the present instance complete sterility was not attempted. Sand analysis again gave no evidence of excretion.

Table II presents the results of associated-growth experiments, the fine quartz sand being used. Except for those in pot No. 8, all the barley plants exhibited signs of intense nitrogen starvation, and analysis confirmed that there had been no appreciable uptake of nitrogen: in so far as they concern the same leguminous varieties these results confirm those of Expt. II. Pot

No. 8 gave what appeared, prior to harvest, to be evidence of excretion but at the time of harvest it was found that a non-viable pea seed (initial nitrogen content 15 mg.) had been left in the sand by oversight. This had decayed and was densely penetrated by the roots of the barley: there is little doubt that it was from this source that the barley plants had gained nitrogen. No

TABLE III

Associated-growth Experiments. Coarse Quartz Sand

Pot No.	Bacillus strain.	Dry wt. of leguminous plants (gm. per pot).	N fixed by leguminous plants (mg.).	N content of associated plants (mg.).	Plants present.
Expt. IV.*	BB	14.8	300	16.0	2 Longpod Broad beans and 1 uninoculated French bean per pot
	"	Similar to Pot No. 1.		17.0	
	"	"	"	19.0	
	Control	—	0	18.0	
	"	—	0	19.0	
Expt. V.†	HX	—	60	3.0	3 Gradus peas and 2 barley per pot.
	"	—	Similar	2.5	
	"	—	"	2.7	
	Control	—	0	2.9	
	"	—	0	2.5	
Expt. VI.‡	HX	4.1	75	8.5	3 Gradus peas and 5 barley per pot.
	GW	4.6	90	6.5	
	HX	4.1	85	6.9	3 Torstag peas and 5 barley per pot.
	GW	4.3	90	5.5	
	HX	6.0	140	6.5	3 Maple peas and 5 barley.
	"	4.4	125	8.0	
	"	2.0	40	11.3§	3 Gladstone peas and 5 barley.
	Control	—	0	7.2	

* April 6-July 31, 1937. Glazed pots containing 3.6 kg. sand. Average initial N content of a French bean seed = 17 mg.

† June 5-August 23, 1937. Glazed pots containing 1.7 kg. sand. N content of 2 barley grains = 1.8 mg. Peas with pods at harvest.

‡ April 20-July 21, 1938. Glazed pots containing 3.6 kg. sand. Initial N content of 5 barley grains = 5.8 mg. Peas flowering or with pods at harvest.

§ See text.

analysis was made of the barley from pot No. 11, but it was quite evident from inspection that there had been no uptake of nitrogen, this being confirmed by a duplicate pot.

Data for various associated cultures grown in the coarse quartz sand are presented in Table III. In Expt. IV uninoculated plants of French bean (*Phaseolus vulgaris* L.) were grown as detectors for excretion from broad beans. It may be of interest to record that although these two leguminous species are placed in different cross-inoculation groups, the French beans

that were associated with inoculated broad beans themselves developed a considerable number of nodules. In no case, however, did the final nitrogen content of the French beans differ significantly from the original seed nitrogen; evidently there had been no fixation of nitrogen within their own nodules (as is quite usual for this particular leguminous species—see Fred, Baldwin, and

TABLE IV

Associated-growth Experiments. Superfine Sand and Soil

Pot No.	Bacillus strain.	N fixed by leguminous plants (mg.).	N content of associated plants (mg.).	Plants present.
Expt. VII.*				
1	9	140	12.0	2 Manchu Soya bean and 5 barley.
2	"	Similar	11.3	
3	HX	68	7.9	
4	Control	0	11.4	2 Torstag pea and 5 barley.
Expt. VIII.†				
1	HX	—	103	6 Concordia pea and 8 barley.
2	"	—	90	6 Torstag pea and 8 barley.
3	BB	—	60	5 Longpod broad bean and 8 barley.
4	Control	—	86	8 barley, no leguminous plants.‡

* April 20-July 23, 1938. Glazed pots containing 1.3 kg. superfine red sand. N content of 5 barley grains = 5.8 mg.

† May 6-August 7, 1938. 8.5 kg. soil per pot.

‡ Accompanying the barley was an equal number of plants of timothy, in connexion with other work.

McCoy, 1932), neither had there been any uptake from the sand. There is thus no evidence for an excretion of nitrogen from the broad bean nodules.

Expt. V (Table III) was run parallel with Expt. I, and, as has been mentioned, it gave no evidence of excretion. Expt. VI concerns four varieties of pea, and here again evidence for excretion is negative. A decayed pea seed was found in pot No. 7 of this experiment at harvest, and hence, no doubt, the somewhat increased nitrogen content of the barley plants. No special significance is attached to the fact that this effect was obtained with 'Gladstone' peas in two experiments. It will be recalled that no evidence of excretion from this variety was obtained in Expt. II (Table I).

The experiments included in Table IV concern other rooting media. In Expt. VII the superfine red sand was employed. It is clear from the control pot that some available nitrogen was initially present in the sand: there is no evidence that the barley of the inoculated pots had access to any other source of nitrogen. In Expt. VIII larger glazed pots, containing an unsterilized, low-nitrogen sandy soil, were used, and in this experiment the plants were kept permanently out-of-doors in a sunny position against a wall, protected from rain by a transparent overhead screen. The leguminous plants made excellent growth and without doubt fixed considerable amounts of nitrogen, though

no determination of fixation was made. Since the control shows that the barley plants were able to secure considerable amounts of nitrogen from the soil, the experiment calls for careful interpretation. The low figure for the nitrogen content of the barley from pot No. 3 is doubtless due to the strong competition for available nitrogen offered by the very vigorous broad beans, and to shading. It was judged that competition in pots Nos. 1 and 2 was much as in the control: on this basis there is no evidence for excretion from 'Torstag' peas, though there may have been slight excretion in the case of the 'Concordia' variety. Experiments of this type will be continued.

IV. DISCUSSION

From the preceding section it is clear that in their sand-culture experiments the authors have been unable to secure evidence of excretion of nitrogenous substances from root nodules, although use was made of ten different varieties of leguminous plants, three grades of sand, and various strains of nodule bacteria. Even when the circumstances of Virtanen's experiments were reproduced as closely as is practicable at another station, excretion was still absent. Thus in some experiments with 'Torstag' pea, in addition to the variety of leguminous plant, the strain of bacillus was the same as is used by Virtanen, the general treatment of the plants was similar and the nature of the sand not markedly different. The few soil-cultures do not permit of a definite statement, but it can at least be said of them that there was no marked excretion.

There are naturally certain differences in climatic factors between the Glasgow and Helsinki experiments: the greatest difference is probably in respect of light intensity. The latter station receives on an average much more direct sunlight during the summer months, as is indicated by the following table. The figures for Helsinki are from Birkeland and Föyn (1932), those for Glasgow being supplied by the *Glasgow Herald* and based upon records taken at a municipal meteorological station.

Hours of Sunshine

		April.	May.	June.	July.	Aug.	Sept.
Helsinki (1911-17)	.	150	250	249	298	204	124
Glasgow (1914-37)	.	127	160	187	149	127	103
" (1938)	.	162	189	170	156	158	74

It will be noted that in July on an average Helsinki receives actually twice as much sunshine as Glasgow. After allowing for the slightly lower altitude of the sun in consequence of difference of latitude (see below), the mean light intensity must be considerably higher in Helsinki during these summer months, in which most of the experiments have been performed. It has been stated that the Glasgow sand-cultures of peas were kept out-of-doors as much as possible, and it is understood that the arrangements at Helsinki also provide for exposure of the plants to the sun in fine weather, so that the above

figures for sunlight are of importance in comparing the experiments at the two stations. It is to be noted that the proximity of buildings prevented the Glasgow plants from receiving all the available sunshine, and also reduced the light intensity somewhat on cloudy days. It remains for future work to reveal the extent to which differences of light intensity are responsible for the discrepant results obtained with peas at the two stations, and to show whether, by growing plants in a position securing a maximum incidence of natural light, it is possible to obtain excretion at the Glasgow station. It may be noted that in the Glasgow soil-cultures, which were kept permanently outside in an unobstructed position securing excellent lighting though still not maximal for the district, there was no clear evidence of excretion. Against the significance of the lower light intensity in Glasgow are certain observations of Wilson and Wyss (1937), who also attempted to explain their own negative results on the basis of differences of light intensity, and also the fact illustrated by the experiments of Wilson and Burton (1938) that very vigorous growth such as might be induced by high illumination is not necessarily accompanied by excretion. These Madison experiments receive further consideration below. It may also be noted that at the Helsinki station successful experiments are carried out in winter, the plants being grown under artificial light from 1,000 W. or 2,000 W. lamps suspended 10–20 cm. above the uppermost leaves of the plants. The light intensity at this level will be of the order of 700 foot-candles in the case of a 1,000 W. lamp; while this is very much inferior to the intensity of direct sunlight, it is chiefly on diffuse light that plants have to rely under the climatic conditions of the Glasgow station. In experiments with Soya beans better growth was obtained under a 1,000 W. lamp in winter than in the natural light of the summer months (Bond, 1938a).

The length of day is also somewhat greater in Helsinki during the summer months; according to Supan (1913) the longest day in Helsinki (latitude 69°) is 18·5 hours, while in Glasgow (lat. 56°) it is 17·5 hours. It may be doubted whether such a small maximum difference of photoperiod will materially affect plant behaviour. There are doubtless some differences of temperature between experiments at the two stations: Virtanen (1938a) has, however, experimented on this point, and he secured marked and approximately equal excretion in peas grown at 10–15° C. and at 20–25° C. The limits of this temperature range were not exceeded in the present experiments. Virtanen has frequently stressed the influence that may be exerted by the properties of the rooting medium, especially its capacity for adsorption, upon excretion. In view of the fairly close similarity as regards particle sizes between the 'fine quartz sand' of the present experiments and the sand used by Virtanen, and the facts that the 'superfine red sand' was still finer and of greater adsorptive capacity, and that soil was used to some extent, it would appear that the absence of excretion in the present experiments was not due to the use of unsuitable rooting media. This point is, however, still under examination.

Further discussion will be assisted by reference to Table V, in which data from Helsinki experiments with 'Torstag' pea, showing varying degrees of excretion, are assembled with figures from Madison and Glasgow experiments on the same variety; data from some experiments with 'Manchu' Soya bean are also included. The comparison of growth (on the basis of dry matter

TABLE V

Data from Various Experiments on Excretion. All Figures are per Plant, and where possible are Averages based on Replicate Pots

Expt. No.	Source.	Dura- (days).	Condition at harvest.	Dry matter of plant (mg.).*	N fixed (mg.).†	Relative fixa- tion.‡	Excre- tion. %
<i>Torstag Pea</i>							
1	Virtanen & von Hausen. ¹	61	Almost ripe.	2100(34)§	52(0.9)§	2.5	22
2	Virtanen, von Hausen & Laine. ²	48	Full bloom.	1650(34)	42(0.9)	2.5	8
3	Virtanen, von Hausen & Laine. ³	48	Full bloom.	1550(32)	46(1.0)	3.0	17
4	Virtanen, von Hausen & Laine. ⁴	38	?	2280(60)	103(2.7)	4.5	44
5	Virtanen, von Hausen & Laine. ⁵	38	?	1820(48)	155(4.0)	8.6	66
6	Virtanen. ⁶	74	Almost ripe.	1780(24)	159(2.1)	9.0	78
7	Virtanen. ⁷	28	?	640(23)	17(0.6)	2.6	83
8	Present paper (Table I).	98	Almost ripe.	2045(20)	47(0.5)	2.5	0
9	Present paper (Table III).	94	"	1370(15)	28(0.3)	2.0	0
10	Wilson & Burton. ⁸	72	"	3900(54)	116(1.6)	2.9	0
11	Wilson & Wyss. ⁹	62	?	—	7(0.1)	—	38
<i>Manchu Soya bean</i>							
12	Wilson & Wyss. ¹⁰	77	Ripe.	3200(42)	126(1.6)	4.0	41
13	Present paper (Table I).	98	Flowering.	4900(50)	90(0.9)	1.8	0
14	Present paper (Table II).	94	"	3230(34)	55(0.6)	1.7	0

¹ J. Agr. Sci., xxv, Table I(i).² Ibid., xxvii, Pt. III, Table VI (ii).³ Ibid., Table VI(i).⁴ Ibid., Table V.⁵ Ibid., Table IV. With barley.⁶ Ann. Agr. Coll. Sweden, v. Table 6. With potato.⁷ Ibid., Table 1.⁸ J. Agr. Sci., xxvii. Expt. VII. With barley.⁹ Soil Science Soc. Proc., 1937, Expt. I. With barley.¹⁰ Ibid., Expt. III. With barley.

* No correction for seed dry matter.

† Includes nitrogen excreted.

‡ See text.

§ Figures in brackets are per day.

formation) and of fixation of nitrogen is complicated by the variation in the stage of development at which harvest was effected in the different experiments. It is, however, obvious that the total amount of growth made by the Glasgow plants during their life-cycle was of the same order as that in the case of the Helsinki experiments. Under Glasgow conditions the life-cycle was, however, more extended, so that growth rates were lower. Incidentally, consideration of Expt. 10 in the table indicates clearly that very vigorous growth is not necessarily accompanied by excretion. The total fixation of

nitrogen in the Glasgow experiments was similar to that in some of the Helsinki series, but again rates were lower; in other Helsinki experiments the fixation was greatly superior. The rapid growth of the plants of Virtanen's experiments is probably due to the more favourable light intensity, and the same explanation may apply to some extent to the more intensive fixation of nitrogen. Expt. 7 in Table V was performed in winter, which no doubt accounts for the low growth and fixation.

That the occurrence of excretion is not dependent on high absolute rates of fixation is indicated by Expt. 7 and still more so by Expt. 11 in Table V, while in Expt. 10, despite vigorous fixation, there was no excretion. In so far as the rate of fixation affects excretion, it seems likely that the rate of fixation relative to the growth of the plant will be of chief importance. One possibility with regard to the events leading up to excretion is that products of fixation are formed more rapidly than they can be utilized in the growth of the plant, which is limited by other factors. Virtanen (1938b) considers this to be the state of affairs which normally induces excretion, while Wilson and Wyss (1937) advanced the same hypothesis in somewhat different terms. It is possible then that Virtanen's conditions favour excretion by promoting a high rate of fixation relative to growth. Is there any evidence that this is the case? For each experiment in Table V the weight of nitrogen fixed during the accumulation of 100 parts by weight of plant dry matter has been calculated; each value is, of course, merely an average for the whole period of an experiment. It will be seen that higher values for this relative fixation are obtained in most of the Helsinki experiments, especially in those showing extensive excretion, than in those of the present writers;¹ in the case of the Soya bean a higher value is obtained for the Madison experiment (No. 12), which gave excretion. In so far as it is the case that excretion is initiated and governed solely by the tendency to a disproportionate rate of fixation, it appears that in these higher values for relative fixation we have the reason for the excretion which was observed. Alternately, however, a high relative fixation may be merely the *result* of an excretion promoted by other factors, such as the presence round the nodules of a rooting medium of high adsorptive properties, or the proximity of the roots of non-leguminous plants; Virtanen (1938a) concludes that both these factors promote excretion under his conditions. For the more rapid removal of the products of fixation from the nodules, which may result from such excretion, may lead to a greater fixation than would otherwise occur. Further, it appears from Virtanen's experiments that excretion in the presence of an associated plant may be so extensive as actually to result in a limitation of the growth of the leguminous plant; such an effect, which was apparent in Expt. 6, Table V, will also produce a high figure for relative fixation. There is, however, the possibility that the higher relative fixation in the Helsinki experiments is one of the causal factors producing the

¹ The marked exception provided by Expt. 7 appears to be due to an unusually low percentage of nitrogen in the plants (1.5 per cent.).

excretion, and that the absence of excretion in the Glasgow experiments is due to an inadequate rate of fixation relative to growth.

It remains for further work to show whether light intensity, which as noted above is lower in Glasgow than at the Helsinki station, is capable of exerting any differential effect upon the processes of growth and fixation. It is possible that as light intensity increases, a higher proportion of carbohydrates becomes available to the bacteria. This might increase relative fixation by stimulating the activity of the bacteria and by leading to a greater development of bacteria and of nodules in relation to the rest of the plant. Virtanen (1938a) has himself suggested that the weight of nodule tissues may influence excretion, through its effect on fixation: unfortunately the present writers did not keep records of nodule weights in their experiments.

It has to be borne in mind that Wilson and Wyss (1937) have also invoked the light factor in an attempt to explain their own negative results in excretion experiments. They suggested, however, that the summer light intensity in Madison (latitude 43°) is too *high* to allow of excretion. On reducing illumination they secured excretion from pea nodules (see Expt. 11, Table V). Judging from the very low fixation in this experiment, it would appear that the light intensity was reduced below the Glasgow level, and the fact that excretion was obtained is obviously at variance with the present writers' suggestion that their own negative results may be due to a light intensity of too low a value.

This discussion has been concerned chiefly with experiments on peas, although the writers' experiments also involved broad beans and Soya beans. There do not appear to be any published observations on excretion from broad bean nodules, but there is no reason to suppose that under suitable conditions excretion would not be obtained from them. The experiments of Wilson and Wyss (see Table V) have proved that excretion can take place from nodules of Soya bean. The plants of their experiments were grown under reduced light, but the absence of data makes comparison of lighting conditions for the Madison and Glasgow experiments impossible. It will be noticed that although the dry matter of the plants was practically the same in Expts. 12 and 14 in Table V, the absolute and hence the relative fixation was more than twice as great in the case of the former experiment. In this connexion there is the same doubt as in the pea experiments as to whether this higher relative fixation is the cause or merely the result of the excretion. It is hoped to provide an answer to some of these problems from further experiments now in progress.

V. SUMMARY

A description is given of experiments carried out at Glasgow designed to detect any excretion of nitrogenous substances from root nodules of different varieties of Soya bean, pea (*Pisum sativum* L.), and broad bean (*Vicia Faba* L.). Various strains of the nodule organism were used for inoculation, while three

different grades of sand were employed as rooting media. In some experiments soil was used. Soya beans were grown in a cool greenhouse, the sand-cultures of pea and broad bean being placed in the open in a sunny position during fine weather. Plants growing in soil-culture were permanently outside in a well-lighted situation. Tests for excretion consisted of analysis of rooting media and of associated plants used as detectors.

Consistently negative results were obtained from the sand-culture experiments, confirming that excretion is not a constant feature of the healthy growth of leguminous plants. In certain experiments conditions were as far as possible similar to those prevailing in the experiments of Virtanen at Helsinki, in which extensive excretion was frequently obtained. Thus the varieties of leguminous plant and the strain of bacterium were the same as used by Virtanen, while the general treatment of the plants was similar and the rooting media of apparently suitable type. In spite of this no excretion was observed. The results of the soil-cultures were inconclusive.

It appears that the most important difference between natural conditions of plant growth at the Helsinki and Glasgow stations is in respect of light intensity. The former station receives considerably more direct sunlight in the summer months than is the case in Glasgow. It remains for further work to show the extent to which the absence of excretion in the Glasgow experiments is connected with this difference in illumination, and whether, by arranging for the maximum possible incidence of natural light on the plants it is possible to secure excretion at the Glasgow station.

Attention is given to the possibility that the conditions of Virtanen's experiments favour a high rate of fixation relative to the growth of the plants, and in this way promote excretion. Calculation shows that Virtanen's experiments are in fact characterized by a high rate of fixation relative to plant growth, but the degree to which this is a cause or merely a result of excretion is uncertain.

We are indebted to members of staff of the Dept. of Agricultural Bacteriology, University of Wisconsin, and of the Laboratory for Nitrogen Fixation, U.S. Dept. of Agriculture, Washington, for opportunities of discussion and to Dr. S. Williams for certain suggestions. One author (J. B.) held a Carnegie Research Scholarship while engaged on this work and the Carnegie Trustees contributed to the cost of materials and of publication. Thanks are due to the officers of the National Institute of Agricultural Botany and to Mr. W. Robb for supplying seeds.

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ADDENDUM

Mechanical analysis of the three grades of sand employed in the above experiments gave the figures included in the following table, British Standard sieves Nos. 16, 30, 60, 150 and 300 being used, with mechanical agitation. The aperture of these sieves are not in all cases simple fractions of a millimetre, and the figure in the first column are given to the nearest second place of decimals.

Particle sizes.	Coarse quartz.	Fine quartz.	Superfine red sand.	Finnish quartz.
	%	%	%	%
>1.00 mm.	2	0	0	0
1.00—0.50 mm.	18	1	0	0
0.50—0.25 mm.	50	39*	0	9
0.25—0.10 mm.	29	68	62†	56
0.10—0.05 mm.	1	1	28	30
<0.05 mm.	0	0	10	5

* All less than 0.3 mm. † All less than 0.15 mm.

Assistance in these analyses was given by the Mining Dept., Glasgow Royal Technical College. The figures for the Finnish sand are based on those given by Wilson and Burton (1938). The slight discrepancy between the above figures for the analysis of the coarse quartz sand and those given previously (Bond, 1938a) is due to the use of square-mesh sieves in the later analysis.

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